

REMARKS

Applicants have carefully considered this Application in connection with the Examiner's Action, and respectfully request reconsideration of this Application in view of the above amendments and the following remarks.

Applicants also wish to thank the Examiner and the supervising Examiner Joseph Weitach and Janet Epsmith for a very helpful telephonic interview held on April 7, 2009

Claims 5, 6, and 8-15 are pending in this application.

I. CLAIM REJECTIONS UNDER 35 USC §103

A. The Examiner has maintained the rejection of Claims 5, 6, and 8-15 under 35 U.S.C. 103(a) as being unpatentable over the Drewett Reference (Drewett et al. *Journal of Biological Chemistry* 276:36, 33444-33451, 2001) in view of the Narula Reference (Narula et al. PNAS Vol. 96, 8144-8149, 1999).

B. The Examiner states that it would have been obvious to one of skill in the art at the time the invention was made to combine the teachings of the Drewett Reference with the Narula Reference to develop a method of diagnosing cardiac disease in an individual comprising the step of identifying cleavage of SRF in at least one cell from a sample from said individual and that the Narula Reference indicates the relationship between apoptosis and heart failure, while the Drewett Reference describes the relationship between Serum Response Factor (SRF) cleavage and apoptosis.

As noted in the Amendment filed 1.7.09, this case presents a situation where a multitude of genes related to apoptosis exist, and where an unclear relationship exists between each specific gene and the apoptotic process. Therefore, the prior art universe is not limited to "a finite number of identified, predictable solutions," as required for obviousness by *KSR*.

Applicants submit that instead of *KSR*, this case is more analogous to a post *KSR* situation presented in *Ortho-McNeil Pharmaceutical Inc. v. Mylan Laboratories Inc.* 86 U.S. P.Q. 2d 1196 (Fed. Cir. 2008), wherein the Federal Circuit held that, there was "clearly not the easily traversed, small and finite number of alternatives that *KSR* suggested might support an inference of obviousness." *Id.* at 1201. Notably in that case, the Federal Circuit also disapproved the expert's "obviousness" testimony by saying "Mylan's expert, Dr. Anderson, simply retraced the path of the inventor with hindsight, discounted the number and complexity of the alternatives, and concluded

that the invention of topiramate [the compound in suit] was obvious.” *Id.* Accordingly, the Federal Circuit determined that the compound claim is not obvious.

The application of these holdings to unpredictable biological and chemical arts including the field of the currently claimed invention, has also been discussed (again post- *KSR*) by the Federal Circuit in *Eisai Co. Ltd. v. Dr. Reddy's Laboratories Ltd.*, 87 U.S.P.Q.2d 1452 (Fed. Cir. 2008), wherein the Court stated that, “[to] the extent an art is unpredictable, as the chemical arts often are, *KSR*’s focus on these ‘identified, predictable solutions’ may present a difficult hurdle because potential solutions are less likely to be genuinely predictable.” *Id.* at 1457.

The current case involves an unpredictable and large number of alternatives with respect to (1) the number of types of cells (both healthy and diseased) which undergo apoptosis, (2) the number of molecules cleaved by caspase-3, and (3) the number of functions of SRF in a cell. Therefore, this case does not present “a finite number of identified, predictable solutions” as required by *KSR*, and the invention cannot be obvious in view of the cited references. Applicants submit the following evidence in support of this argument.

1. Heart Failure Does Not Always Involve Apoptosis

The Examiner has stated that the Narula Reference provides a linkage between apoptosis and heart failure, and therefore discloses an element of the current claims. However, heart failure does not always include apoptosis, and therefore the Narula Reference must be seen as one teaching amidst a sea of other references which teach a type of healthy or diseased tissue wherein apoptosis has or has not been detected.

Applicants are submitting evidence herewith that several types of heart failure are not associated with apoptosis at all. Specifically, the Kang Reference (Kang et al. *Circulation Research* 86:1107-1113, 2000, **Exhibit 1**) teaches on p. 1109, second full paragraph, that “...not all models of heart failure are associated with apoptosis.” See also Table 2, which lists end-stage heart failure on LV assist device, spontaneous hypertensive rat (SHR) transition to heart failure, viral myocarditis, cardiac allograft rejection, FasL overexpression, dominant-negative CREB transcription factor, and TNF- α overexpression as heart failure models which are not associated with increased apoptosis. Clearly, the Kang Reference would teach away from the detection of apoptosis to diagnose heart disease.

Moreover, the Kang Reference teaches on p. 1107 that, “(i)t is now clear that apoptosis is an important aspect of normal organ development and cellular regulation and that it plays a role in a wide variety of physiological and pathological conditions.” Therefore, apoptosis also occurs in healthy cells which are not undergoing heart failure or any other type of disease. Taken together, these citations show that apoptosis is not an unambiguous marker of heart failure, since not all failing hearts undergo apoptosis, and since healthy cells undergo apoptosis under certain circumstances.

The Cohen Reference (Cohen, GM *Biochemical Journal* 326:1-16, 1997, **Exhibit 2**) states that, “(a)poptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in chemical-induced cell death. Inappropriate apoptosis is implicated in many human disease, including neurodegenerative diseases such as Alzheimer’s disease and Huntington’s disease, ischaemic damage, autoimmune disorders and several forms of cancer” (citations removed). This clearly shows that apoptosis is not limited to heart cells, or limited to diseased cells. Therefore, the teachings of the Narula Reference would not be sufficient to narrow the search for a method of diagnosing heart disease to the detection of apoptosis given that it is one reference among an enormous collection of literature which discloses apoptosis in various cell types, both healthy and diseased.

Given these complicating factors, the relationship between apoptosis and heart disease clearly does not present the easily traversed, small and finite number of alternatives required by *KSR* for obviousness.

2. Caspase-3 Is Not Always Associated With SRF Or Heart Disease

The Examiner has stated that the Drewett Reference teaches a linkage between SRF fragments generated by caspase-3 cleavage and apoptosis, and that this would have led one of skill in the art to use SRF cleavage to measure apoptosis in an effort to detect heart disease.

Applicants respectfully submit that the Examiner is oversimplifying the role of caspase-3 in the apoptotic cascade using hindsight reasoning based on the disclosure of the current specification. Caspase-3 cleaves numerous molecules, as described in the Cohen Reference, p. 11, first full paragraph, which states that, “(c)aspase-3 is responsible, either wholly or in part, for the proteolytic cleavage of a large number of substrates during apoptosis, including PARP, DNA-dependent protein

kinase (DNA-PK), U1-70 kDa, heteronuclear ribonucleoproteins C1 and C2, sterol regulatory binding proteins, D4-GDP dissociation inhibitor, huntingtin, and almost certainly retinoblastoma protein (Rb).” See also Table 3, listing targets of caspase-3 during apoptosis, none of which is SRF. Therefore, it is clear that caspase-3 targets a broad number of molecules for cleavage, and even if one were to select apoptosis as a means for diagnosing heart disease despite the discussion above, that selection would not implicate SRF above any of the other molecules as a candidate for detecting any particular form of apoptosis in heart cells.

Moreover, the Cohen Reference teaches that caspase-3 may be an important mediator of apoptosis in the immune system (p. 5, last paragraph) and is critical for morphogenic cell death in the mammalian brain (p. 7, second full paragraph). These teachings show that caspase-3 activity is by no means limited to the heart or to cardiac cells.

Therefore, the relationship between caspase-3 and SRF, as well as the relationship between caspase-3 and heart disease, clearly does not present the easily traversed, small and finite number of alternatives required by *KSR* for obviousness.

3. SRF Is Not Always Associated With Apoptosis Or Heart Disease

Similarly, SRF is also found in a variety of apoptotic and non-apoptotic cells in a variety of tissues, and plays a role in a number of pathological and non-pathological conditions. The Selvaraj Reference (Selvaraj A, and Prywes, R, *BMC Molecular Biology* 5:13, 2004) teaches in the abstract that, “(s)erum Response Factor (SRF) is a transcription factor that is required for the expression of many genes including immediate early genes, cytoskeletal genes, and muscle-specific genes.” Therefore, there would be no way for one of skill in the art to know that SRF cleavage in any particular cell was a result of normal apoptosis or of apoptosis associated with disease (e.g. heart disease). In addition, since SRF occurs in a multitude of tissue types, there would be no suggestion or motivation to use it particularly for investigating apoptosis in heart tissue.

As described in the Amendment filed 1.7.09, a skilled artisan might have chosen any one of the many genes involved in the apoptotic cascade for further investigation, and many of these would not have turned out to be relevant for the particular form of apoptosis which occurs in heart cells which are experiencing heart disease (as opposed to any other cell type, or cells which are undergoing normal differentiation). The author of the Narula Reference describes the state of the art

relating to the understanding of the apoptotic cascade in human disease in the last full paragraph of the second column of page 8144, stating that:

“(a)lthough sequential activation of the apoptotic cascade has been well characterized in nematodes, in cell culture, and in cell free systems, **little information is available in human diseases.**
[Emphasis added]”

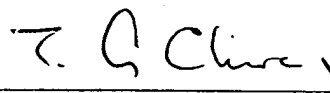
Therefore, none of the possible avenues for research described above provides an easily traversed, small and finite number of alternatives, as required by *KSR* for obviousness. The complex and incompletely understood pathway of molecules in the apoptotic cascade in human disease, and the fact that the cascade varies with cell type and disease state in the cell, means that even if a skilled artisan had chosen SRF and detection of the particular region of SRF described here, there would have been no assurance that it would have served as a useful predictive marker for heart disease.

II. CONCLUSION

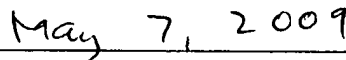
Applicants respectfully submit that, in light of the foregoing Amendment and comments, Claims 5, 6, and 8-15 are in condition for allowance. A Notice of Allowance is therefore requested.

If the Examiner has any other matters which pertain to this Application, the Examiner is encouraged to contact the undersigned to resolve these matters by Examiner's Amendment where possible.

Respectfully submitted,



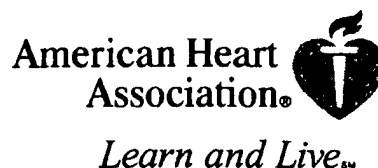
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Apoptosis and Heart Failure : A Critical Review of the Literature

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This MiniReview is part of a thematic series on **Apoptosis in the Cardiovascular System**, which includes the following articles:

Apoptosis and Heart Failure: A Critical Review of the Literature

Vascular Cell Apoptosis in Remodeling, Restenosis, and Plaque Rupture

Apoptosis During Cardiovascular Development

Myocyte Apoptosis in Ischemic Heart Disease

Endothelial Cell Apoptosis in Angiogenesis and Vessel Regression

Richard Kitsis, Editor

Apoptosis and Heart Failure A Critical Review of the Literature

Peter M. Kang, Seigo Izumo

When the concept of apoptosis was introduced in the 1970s,¹ it attracted only limited attention. However, less than two decades ago, Horvitz and colleagues²⁻⁴ identified its essential genetic components in the roundworm, *Caenorhabditis elegans*, and apoptosis emerged as a significant research front. The explosion of knowledge that took place is represented by the accumulation of >25 000 studies in the last 5 years alone. It is now clear that apoptosis is an important aspect of normal organ development and cellular regulation and that it plays a role in a wide variety of physiological and pathological conditions. However, there is still much debate and controversy concerning the role of apoptosis in heart failure. To address the issues of its presence in, significance for, and overall contribution to heart failure, we will review the currently available literature and then discuss its implications for future research and treatment strategies in heart failure.

Evidence of Apoptosis in Animal and Human Models of Heart Failure

The etiology of heart failure involves multiple agents and conditions,⁵ but the progressive loss of cardiac myocytes is one of the most important pathogenic components. During the past few years, there has been accumulating evidence in both human and animal models suggesting that apoptosis may be an important mode of cell death during heart failure (Table 1). Therefore, the possibility of limiting cardiac myocyte loss by

inhibiting apoptosis has potentially important implications in the treatment of heart failure.

The numerous animal models of heart failure encompass a spectrum of species and a variety of apoptotic inducers.⁶⁻²⁸ These studies suggest that the rate of occurrence of apoptosis can vary widely and depends on the model used and the area at risk examined. For example, in acute ischemia and reperfusion, apoptosis can be high as 14% in the area at risk.²⁵ In contrast, the rate of apoptosis associated with chronic stimuli, such as pressure overload, is <1% in nontransgenic models when measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining.^{14,17} But even though the rate of apoptosis in heart failure is relatively low in absolute numbers, it is significantly higher than that in the normal heart, which has essentially negligible baseline apoptosis.

In human heart failure, the data are limited to postmortem samples or tissue samples from patients undergoing heart transplantation.²⁹⁻⁴¹ Although the initial studies reported unrealistically high levels of apoptosis in failed hearts (as much as 35%),^{40,41} more recent studies showed apoptosis rates of <1% (TUNEL-positive cells) during heart failure.^{31,35,38} The most common forms of heart failure associated with apoptosis are idiopathic dilated cardiomyopathy and ischemic cardiomyopathy, but apoptosis has been observed in other forms of heart failure as well.^{36,39,41}

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TABLE 1. Heart Failure Models Associated With Increased Apoptosis in Heart

Model	Species	TUNEL	Comments
Animal model			
Ligand-activatable caspase-8 overexpression	Tg mice	NR	Inhibition of cardiac myocyte apoptosis by broad-spectrum caspase inhibitor ⁵
G _{αs} overexpression	Tg mice	0.6%	Decreased myocardial damage and preserved cardiac function by β-adrenergic blocker ^{6,7}
Gp130 knockout + TAC	Tg mice	3% (baseline)	Apoptosis rate increased to 34% in Gp130 knockout ⁸
Cardiac allograft rejection in NOS ₂ ^{-/-} mice	Tg mice	2.5× control	Antiapoptosis model (decreased caspase-3 activity, p53, and Bcl-xL, increased Bcl-2/Bax ratio ⁹)
G _{αs} overexpression + pregnancy	Tg mice	26%	Increased p38 and JNK ¹⁰
IGF-1 overexpression + coronary ligation	Tg mice	4.2× control	IGF-1 overexpression decreased apoptosis and infarct size ¹¹
Viral myocarditis (3 mouse strains)	Mice	NR	Increased apoptosis and ANF in only 1 mouse strain ¹²
Pressure overload by TAC	Rat	LVH 0.08% LVD 0.15%	Increased Bax, decreased Bcl-2 ¹³
MI model	Rat	2× control	Increased JNK and lipid peroxidation activity ¹⁴
Ischemia/reperfusion	Rat	11.1%	Apoptosis rate decreased to 3.1% by caspase inhibitor, zVAD.fmk ¹⁵
Falling spontaneous hypertensive rats	Rat	0.04%	Decreased apoptosis to 0.009% by ACE inhibitor ¹⁶
MI model	Rat	0.07%	Decreased Bcl-2, increased Bax ¹⁷
Pacing-induced DCM	Dog	4× control	IGF-1 partially block apoptosis ¹⁸
Acute chagasic myocarditis	Dog	NR	EM findings of apoptosis ¹⁹
Ischemic HF by intracoronary microembolism	Dog	0.27% (baseline)	Decrease rate of apoptosis to 0.08% with ACE inhibition ²⁰
Ischemic HF by intracoronary microembolism	Dog	0.41%	Positive EM for apoptosis ²¹
Pacing-induced DCM	Dog	0.37%	Increased PCNA and Fas ²²
Ischemia/reperfusion	Rabbit	13%	Inhibition of apoptosis and infarct size by caspase inhibitor ²³
Ischemia/reperfusion	Rabbit	14.7%	Decreased apoptosis by carvedilol treatment (3.4%) ²⁴
MI model	Pig	0.02%	Hibernating myocardium ²⁵
Ischemic HF by intracoronary microembolism	Sheep	NR	Increased DNase 1, caspase-2, -3 activities ²⁶
Coxsackievirus myocarditis	Orangutan	NR	Case report ²⁷
Human samples (No. of patients)			
End-stage HF + LV assist device (8 DCM, 2 IHD)	NA	NR	Increased Bcl-xL, decreased pro-ANP, DNA fragmentation with LV assist device placement ²⁸
End-stage HF (21)	NA	NR	Decreased serum eNOS expression ²⁹
End-stage HF for transplant (7 females, 12 males)	NA	35× (female), 85× (male) control	Necrosis increased by ×13 (female) and ×27 (male) vs controls ³⁰
Ischemic HF (7)	NA	NR	Increased DNA fragmentation in areas with increased Fas staining ³¹
End-stage HF for transplant	NA	0%–3.9% IHD, 0%–30.5% DCM	Different rate of apoptosis for different causes of heart failure ³²
End-stage HF for transplant (16)	NA	NR	Presence of cytosolic cytochrome c and increased caspase-3 activity ³³
Explanted hearts (21 DCM, 14 IHD)	NA	0.075% IHD, 0.119% DCM	Increased Bcl-2 ³⁴
Acromegalic cardiomyopathy (10)	NA	0.26%	Case series ³⁵
Myocarditis (16)	NA	0.36%	Increased soluble FasL ³⁶
End-stage HF for transplant (36)	NA	0.23%	Increased Bcl-2, no change in Bax ³⁷
Hypertrophic CM (1)	NA	NR	Case report ³⁸
End-stage HF (4 DCM, 3 IHD)	NA	5%–35%	Increased DNA fragmentation ³⁹
Arrhythmogenic RV dysplasia (6)	NA	0%–28%	Increased caspase-3 expression ⁴⁰

ACE indicates angiotensin-converting enzyme; ANF, atrial natriuretic factor; ANP, atrial natriuretic peptide; DCM, dilated cardiomyopathy (CM); EM, electron microscopy; eNOS, endothelial constitutive NO synthase; HF, heart failure; IGF-1, insulin-like growth factor-1; IHD, ischemic heart disease; JNK, c-Jun N-terminal kinase; LVD, left ventricular (LV) dilatation; LVH, left ventricular hypertrophy; MI, myocardial infarction; NA, not applicable; NOS, NO synthase; NR, not reported; PCNA, proliferating cell nuclear antigen; RV, right ventricular; TAC, transverse aortic constriction; and Tg, transgenic.

TABLE 2. Heart Failure Models Not Associated With Increased Apoptosis in Heart

Model	Species	TUNEL	Comments
End-stage HF on LV assist device	Human	<0.1%	Increased Bcl-2 after LV assist device insertion ⁴¹
SHR transition to HF	Rat	0.6%	TUNEL + mostly in nonmyocytes, increased Bcl-xL ⁴²
Viral myocarditis	Mice	Nearly absent	Positive DNA laddering but TUNEL + cells were infiltrating mononuclear cells ⁴³
Cardiac allograft rejection	Mice	<1%	TUNEL + cells were mostly cellular infiltrates ⁴⁴
FasL overexpression	Tg mice	NR	Mild leukocytic infiltration and hypertrophy ⁴⁵
Dominant-negative CREB transcription factor	Tg mice	NR	DCM but no increase in TUNEL and DNA laddering ⁴⁶
TNF- α overexpression	Tg mice	Rare	TUNEL + cells were mostly nonmyocytes ⁴⁷

CREB indicates cAMP-responsive element-binding protein; SHR, spontaneous hypertensive rat.

Problems With Interpreting the Presence of Apoptosis in Heart Failure

Despite the wealth of published data, there are still many controversies surrounding the presence and the significance of apoptosis in heart failure. These controversies stem largely from the limitations of the technique used to detect apoptosis and the difficulties in translating these findings to the ultimate significance of apoptosis in heart failure.

Acute insults, such as myocardial infarction and ischemia/reperfusion, and chronic conditions, such as ischemic and dilated cardiomyopathies, have been linked to increased apoptotic cell death in human and animal hearts.^{38,40,42} But not all models of heart failure are associated with apoptosis^{43–49} (Table 2). Notably, the presence of apoptosis in inflammatory myocarditis is a controversial issue. In viral myocarditis, increased TUNEL staining is associated mostly with infiltrating mononuclear cells or noncardiac myocytes rather than cardiac myocytes.^{44–46,49} In contrast, autoimmune myocarditis is associated with increased TUNEL staining in cardiac myocytes as well as in lymphocytes.⁵⁰ Although more studies are needed, it is likely that the presence of apoptosis depends on the model system.

The specificity of TUNEL staining, which is the most widely used method to detect apoptosis in the heart, has also been challenged. Using the electron microscopic TUNEL method, Fujiwara's group^{51,52} showed that positive TUNEL staining is associated not only with apoptotic myocytes but also with oncotic (necrotic) myocytes or even viable myocytes that are undergoing DNA repair. Because the rate of apoptosis is generally very low in normal heart as well as in heart failure, a high false-positive rate severely limits the interpretation of TUNEL-positive cells. On the other hand, there are other limitations of the TUNEL staining that will significantly underestimate the true incidence of apoptosis and thus obscure its significance in heart failure. For example, because the apoptotic process is transient, the window of opportunity for detecting apoptotic cells by use of TUNEL staining will also be transient. In lymphocytes, the TUNEL-positive period is generally <12 hours. If the same holds true for cardiac myocytes, TUNEL staining may markedly underestimate the true prevalence of apoptosis in heart failure, which usually occurs over many months or years. Moreover, the rate of apoptosis may be variable and may depend on the disease stage. Most of the human studies to detect apoptosis have been performed in patients undergoing heart transplant, who are at the advanced stage of the disease. It is possible that

samples obtained from these patients represent a "burnt-out" state, characterized by minimal ongoing apoptosis, much like a battlefield days after the fighting has ended.

The definition of apoptosis, compared with necrosis, also has been a subject of controversy. Necrosis is an unregulated process leading to cell demise, but apoptosis is ordered and regulated. Therefore, apoptosis can be, at least in theory, prevented or inhibited if intervention occurs at an early stage. A number of studies have attempted to distinguish apoptosis and necrosis under various conditions with the use of several methodologies.^{17,31,53} Because necrosis, in contrast to apoptosis, is characterized by inflammation and the release of intracellular contents that are toxic to neighboring cells, significantly different consequences on cardiac hemodynamics may result. However, at this time, it is unclear whether necrosis and apoptosis are 2 distinct and independent cell death pathways. It has been suggested that the difference between apoptosis and necrosis is in the level of intracellular ATP present and that the cell that is undergoing apoptosis can be made to undergo necrosis by intracellular ATP depletion.^{54,55} Because the consequence of cell death by either mode is ultimately cell loss, the most important issue from a therapeutic standpoint is whether the cell loss can be attenuated. Thus, rather than a strict distinction between apoptosis and necrosis, whether cell death ultimately can be inhibited or not may prove to be a more important distinction from the clinical standpoint.

Because of the limitations associated with TUNEL staining and the difficulties of interpreting these findings (however well done), the use of TUNEL alone to detect the presence of apoptosis is not sufficient to define the role of apoptosis in heart failure. We need more studies using *in vitro* models of cardiac myocyte apoptosis to decipher and to understand the molecular mechanism of apoptosis in cardiac myocytes. In addition, we need "interventional studies" using transgenic and cardiac-specific gene knockout mice models to study the consequences of genetic manipulation of proapoptotic and antiapoptotic genes *in vivo*. These should be complemented by studies in larger animal models (eg, pigs or dogs) that can mimic human clinical conditions much better than murine models, as well as by pharmacological studies to modulate apoptosis.

Models of Heart Failure From Cardiac Apoptosis

Recently, animal models of heart failure incorporating transgenic technology have confirmed that myocyte apoptosis

itself is sufficient to induce heart failure.⁶ Kitsis et al⁶ generated transgenic mice with cardiac-specific overexpression of ligand-activatable cysteine aspartate proteinase (caspase-8), which is an artificial (or engineered) fusion protein consisting of the FK506 binding protein and the catalytic domain of caspase-8. Transgenic mice expressing this construct appeared normal at birth, but administration of the divalent dimerizer FK1012 activated caspase-8 and -3, resulting in overwhelming cardiac myocyte apoptosis and rapid death of the animal. Perhaps not surprisingly, even in the absence of a dimerizer, adult mice with high expression of the protein manifest spontaneous cardiac myocyte apoptosis, leading over time to a lethal dilated cardiomyopathy. These changes were ameliorated by a broad-spectrum caspase inhibitor (R. Kitsis, personal communication, 2000). Although it is not clear whether endogenous caspase-8 is an important regulator of apoptosis in heart, the study of Kitsis et al⁶ nevertheless demonstrates that the induction of apoptosis can be achieved in the heart. It also shows that slow induction of apoptosis alone can result in dilated cardiomyopathy.

Another mouse model of heart failure uses cardiac-specific knockout of gp130, a common subunit of the interleukin-6 family of cytokine receptors that have been shown to promote cell survival in the presence of an apoptotic stimulus *in vitro*.⁹ Under baseline conditions, these mice showed a grossly normal phenotype with normal cardiac structure and function. However, when gp130 knockout mice were exposed to acute pressure overload by surgical constriction of the transverse aorta, they developed significant cardiac apoptosis ($\approx 34\%$), and $>90\%$ died by dilated cardiomyopathy in a few weeks.⁹ In contrast, normal wild-type mice exposed to similar pressure overload developed compensatory cardiac hypertrophy without heart failure. Of particular interest is that aortic-banded gp130 knockout mice did not develop hypertrophy. Because gp130 has been shown to provide important survival signals in the cardiac myocyte during cardiac hypertrophy via cardiotrophin-1,⁵⁶ this model provides important clues to the relationship between hypertrophy and apoptosis. For example, for adaptive cardiac hypertrophy to occur in response to mechanical stress, it is necessary to have antiapoptotic or survival factors, such as gp130, present in heart. The notion that cardiac hypertrophy is a favorable adaptation to stress and that hypertrophied cells can be more resistant to an apoptotic stimulus is also supported by other transgenic models of cardiac hypertrophy. Overexpression of calcineurin confers a protective effect on cardiac myocytes both *in vitro* and *in vivo* when they are exposed to apoptotic stimuli.⁵⁷ Also, cardiac hypertrophy by overexpression of insulin-like growth factor-1 in animals has been shown to limit infarct size by limiting apoptotic cell death.^{12,19}

On the other hand, the overexpression of heterotrimeric G proteins, such as $G_{\alpha o}$ or $G_{\alpha s}$, may promote apoptosis in cardiac myocytes.^{7,8,11} For example, transgenic mice with overexpression of $G_{\alpha s}$ signaling develop compensatory hypertrophy at baseline. However, when transgenic females become pregnant, they develop lethal dilated cardiomyopathy, resembling human peripartum cardiomyopathy, within 1 week after delivery.¹¹ TUNEL staining of the heart revealed markedly increased levels of apoptosis ($\approx 26\%$). Also, $G_{\alpha s}$ overex-

pression results in increased sensitivity to apoptotic stimulation and leads to cardiomyopathy.⁷ This was confirmed by blocking the β -adrenergic receptor, which prevented myocyte damage, decreased cardiac apoptosis, and preserved cardiac function in $G_{\alpha s}$ transgenic mice.⁸ In addition, other important hypertrophic signaling molecules, such as angiotensin II, have also been shown to promote apoptosis *in vitro*,⁵⁸ and several studies show that administration of angiotensin-converting enzyme inhibitors blocks cardiac apoptosis *in vivo*.^{17,21}

These models of heart failure in mice demonstrate that apoptosis does occur during heart failure and could play a significant role in the development of heart failure in certain settings. However, whether hypertrophy renders cardiac myocytes more sensitive or resistant to apoptosis is still controversial. Some hypertrophic signaling factors, such as cardiotrophin-1 via gp130, insulin-like growth factor-1 via phosphoinositide-3 kinase, and calcineurin via the nuclear factor of activated T cells, seem to be protective. On the other hand, hypertrophic signaling via heterotrimeric G proteins and angiotensin II seems to render cardiac myocytes more sensitive to apoptosis. These findings are important because they provide possible strategies to modulate cardiac apoptosis. However, we believe further studies, especially during the transition from compensated hypertrophy to heart failure, are needed to better understand the complex and delicate balance that exists among hypertrophy, apoptosis, and heart failure.

Regulation of Cardiac Apoptosis

The major apoptotic pathway is initiated by the release of cytochrome *c* from mitochondria in response to an apoptotic stimulus. Released cytochrome *c*, in the presence of dATP, forms an activation complex with apoptotic protein-activating factor-1 and caspase-9 that activates downstream caspases to execute the final morphological and biochemical alterations.^{59–63} This pathway is tightly regulated by a group of antiapoptotic proteins, such as Bcl-2, and proapoptotic proteins, such as Bax^{64,65}; further regulation occurs downstream by various inhibitors of caspases.⁶⁶ There is recent evidence to suggest that cardiac myocytes also use a mitochondria-dependent apoptotic pathway.^{67–69} Cytosolic cytochrome *c* and the activation of caspases have been observed in both human and animal models of heart failure.^{10,27,34} The level of Bcl-2 is upregulated soon after acute coronary occlusions, especially in the salvageable myocardium,^{53,70,71} but is decreased after chronic heart failure by pressure overload.¹⁴ Also, the overexpression of Bcl-2 in the heart effectively reduces myocardial reperfusion injury by reducing cardiac myocyte apoptosis.⁷² Moreover, some studies suggest that the balance between Bcl-2/Bax may be important in the increased rate of apoptosis in cardiac myocytes.^{10,14,18,23} These findings are supported by the reversal of the Bcl-2/Bax ratio in the heart after left ventricular assist device placement.²⁹

It has been suggested that the cardiac myocyte could also use an alternative apoptotic pathway that activates downstream caspases via "death receptors" (eg, Fas, tumor necrosis factor [TNF] receptor) and caspase-8.^{32,37} Expression of the death receptor Fas is upregulated in cardiac myocytes during myocardial ischemia and heart failure,^{23,25,37,53} and

increased levels of soluble Fas ligand and TNF- α have been reported in patients with end-stage heart failure.⁷³ Also, in immune-mediated cardiomyopathies, cardiac apoptosis is associated with an augmented Fas/FasL system.⁵⁰ However, cardiac-specific overexpression of both TNF- α and FasL did not result in increased cardiac myocyte apoptosis.^{47,49} Therefore, we speculate that although a death receptor-mediated pathway may be important in certain situations, notably in immune-mediated heart failure, this may not be the main pathway in more common forms of heart failure, such as ischemic and dilated cardiomyopathy.

Is There a Potential for Antiapoptotic Therapy for Heart Failure?

Several strategies can be used to inhibit apoptosis in heart, and various agents and factors have been shown to be effective in experimental models.⁷⁴ For example, caspase inhibitors significantly decrease apoptosis in the area at risk, with subsequent reduction in infarct size in rat hearts during experimentally induced ischemia and reperfusion.^{16,24} Furthermore, the long-term beneficial effects of angiotensin-converting enzyme inhibitors and carvedilol in the treatment of heart failure may involve, at least in part, inhibition of cardiac apoptosis.^{17,21,25}

Even though the therapeutic targeting of apoptotic pathways has potential in the treatment of heart failure, several important questions still need to be answered. First, it has not yet been shown whether inhibition of apoptosis could delay or prevent the development of heart failure. It is possible that inhibiting apoptosis may simply result in the activation of another mode of cell death, such as necrosis, which may have more deleterious effects on neighboring cells and ultimately a worse outcome. Although the early studies on animal models of heart failure have been encouraging, the long-term consequences of inhibiting apoptosis in the heart are not known. Second, the safety of antiapoptotic therapy has not been tested. Apoptosis is needed for the normal functioning of other cell systems, such as the immune system, and an excessive inhibition of apoptosis is associated with lymphoma or autoimmune disorders. Therefore, the chronic systemic inhibition of apoptosis may have significant deleterious consequences in noncardiac organs. Third, antiapoptotic therapy for heart failure may not apply to all types of heart failure. We speculate that an antiapoptotic strategy for heart failure due to persistent pressure overload will remain controversial for a while, because chronic and complete inhibition of apoptosis may be very difficult to achieve with the current repertoire of drugs. The role of antiapoptotic therapy in heart failure associated primarily with inflammation (eg, viral myocarditis) may also remain controversial because the removal of virally infected cells is likely to be a necessary step toward recovery. The most ideal conditions for antiapoptotic intervention, in our opinion, occur in transient and acute insults, such as reperfusion. During reperfusion, cardiac myocyte apoptosis occurs at a high rate during a defined time period; thus, a short treatment period may be highly effective. Moreover, a short therapeutic course has the additional benefit of minimizing the possible deleterious side effects arising in other organ systems.

Conclusion

It is clear that apoptosis plays an important role in a variety of physiological and pathological states. However, in the cardiovascular system, we have only begun to clarify the role of apoptosis and to start exploring the therapeutic potential associated with its inhibition. Still more work is necessary to understand the molecular mechanisms that govern these processes and the significance of apoptosis in heart failure. Apoptosis must be demonstrated by multiple criteria, not just TUNEL staining alone. Genetic interventional studies should be explored further by use of mouse models, but pharmacological studies using large animal models should be encouraged. The initial analytical work must be carried out in well-defined experimental frameworks that are tissue-targeted and time specific, with clear quantitative end points. Only then will we be able to conduct meaningful human studies to answer whether the inhibition of apoptosis in heart failure will translate into clinical benefit.

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KEY WORDS: apoptosis ■ heart failure ■ treatment ■ humans ■ animals

REVIEW ARTICLE

Caspases: the executioners of apoptosis

Exhibit 2

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Apoptosis is a major form of cell death, characterized initially by a series of stereotypic morphological changes. In the nematode *Caenorhabditis elegans*, the gene *ced-3* encodes a protein required for developmental cell death. Since the recognition that CED-3 has sequence identity with the mammalian cysteine protease interleukin-1 β -converting enzyme (ICE), a family of at least 10 related cysteine proteases has been identified. These proteins are characterized by almost absolute specificity for aspartic acid in the P₁ position. All the caspases (ICE-like proteases) contain a conserved QACXG (where X is R, Q or G) pentapeptide active-site motif. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide (prodomain) together with one large and one small subunit. The crystal structures of both caspase-1 and caspase-3 show that the active enzyme is a heterotetramer, containing two small and two large subunits. Activation of caspases during apoptosis results in the cleavage of critical cellular substrates, including poly(ADP-ribose) poly-

merase and lamins, so precipitating the dramatic morphological changes of apoptosis. Apoptosis induced by CD95 (Fas/APO-1) and tumour necrosis factor activates caspase-8 (MACH/FLICE/Mch5), which contains an N-terminus with FADD (Fas-associating protein with death domain)-like death effector domains, so providing a direct link between cell death receptors and the caspases. The importance of caspase prodomains in the regulation of apoptosis is further highlighted by the recognition of adapter molecules, such as RAIDD [receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain]/CRADD (caspase and RIP adapter with death domain), which binds to the prodomain of caspase-2 and recruits it to the signalling complex. Cells undergoing apoptosis following triggering of death receptors execute the death programme by activating a hierarchy of caspases, with caspase-8 and possibly caspase-10 being at or near the apex of this apoptotic cascade.

INTRODUCTION

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in chemical-induced cell death [1–3]. Inappropriate apoptosis is implicated in many human diseases, including neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer [4,5]. Apoptosis is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations [1,6]. Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by dramatic stereotypic morphological changes in cell structure [7], suggesting the presence in different cells of a common execution machinery [8]. Apoptosis is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum (frequently in a subplasmalemmal distribution), a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells, so preventing an inflammatory response [1] (Figure 1). The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA [9], recognized as a 'DNA ladder' on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of

apoptosis. These DNA ladders are derived from large fragments of DNA of 30–50 and 200–300 kbp, which may in terms of higher-order chromatin structure represent loops and rosettes of DNA [10–12]. Internucleosomal cleavage of DNA now appears to be a relatively late event in the apoptotic process, which in some models may be dissociated from early critical steps [13,14]. Nevertheless, its measurement is simple and it is often used as a major criterion to determine whether a cell is apoptotic.

Whereas early studies concentrated on the role of nucleases in apoptosis, more recently a role has been proposed for a number of different proteases, including serine proteases, calpains and proteasomes (reviewed in [15–18]). Most attention has focused on the interleukin-1 β (IL-1 β)-converting enzyme (ICE)-like proteases, due partly to the enormous progress made by Horvitz and his colleagues in understanding programmed cell death in the nematode *Caenorhabditis elegans*. During normal development, 131 cells of the 1090 cells generated die by apoptosis [2]. Two genes, *ced-3* and *ced-4*, are vital for cell death in *C. elegans*, while the *ced-9* gene antagonizes their function and prevents cell death [19]. The CED-9 protein bears sequence similarity to mammalian Bcl-2, which acts to prevent cell death in mammals. No mammalian homologue of CED-4 has yet been identified. The CED-3 protein bears marked sequence similarity to, and identity with, mammalian ICE [20]. This seminal finding, together with the observation that overexpression of ICE induces apoptosis, suggests that ICE may play a key role in the induction of apoptosis [20,21]. Further evidence supporting a critical role for

Abbreviations used: Ac, acetyl; AMC, 7-amino-4-methylcoumarin; CPP32, 32 kDa cysteine protease; CRADD, caspase and RIP adapter with death domain; DED, death effector domain; DNA-PK, DNA-dependent protein kinase; DNA-PK_{cs}, catalytic subunit of DNA-PK; FADD/MORT1, Fas-associating protein with death domain; ICE, interleukin-1 β -converting enzyme; ICH, *Ice* and *ced-3* homologue; ICH-1_L and ICH-1_S, long and short isoforms respectively of ICH-1; IL-1 β , interleukin-1 β ; PARP, poly(ADP-ribose) polymerase; RAIDD, RIP-associated ICH-1/CED-3-homologous protein with a death domain; Rb, retinoblastoma protein; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR-1, TNF receptor; TRADD, TNFR-1-associated death domain protein; U1-70 kDa, 70 kDa protein component of the U1 small nuclear ribonucleoprotein; Z-VAD.FMK, benzylloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone.

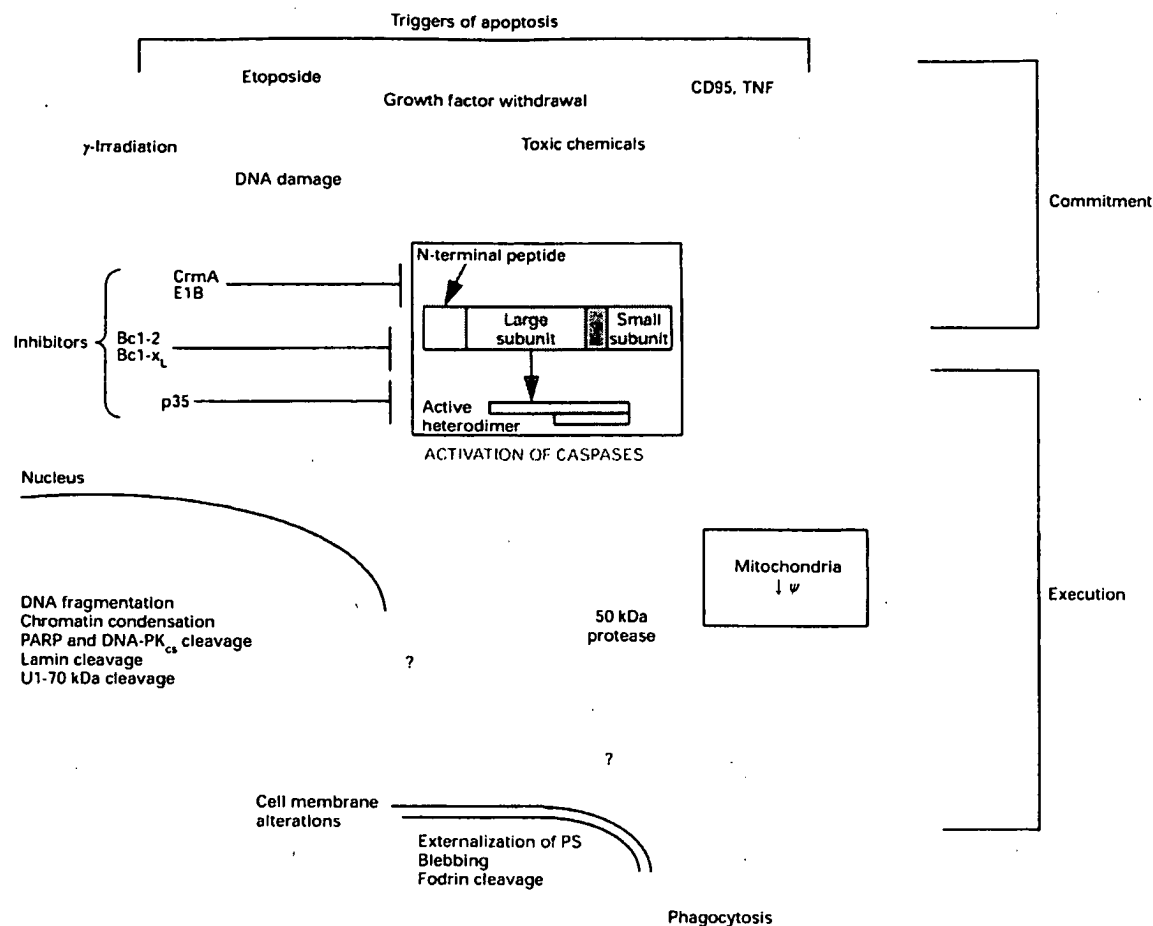


Figure 1 Activation of caspases represents a major control point in apoptosis

Apoptosis involves an initial commitment phase followed by an execution phase. Activation of the caspases results in nuclear, plasma-membrane and mitochondrial changes. The latter include the release of a 50 kDa protease, which may be responsible for some of the cellular changes associated with apoptosis. ψ , mitochondrial membrane potential; PS, phosphatidylserine. See the abbreviations footnote for other definitions.

Table 1 Members of the caspase family

Caspases-3, -7 and -9 have only one cleavage site between the large and small subunits, whereas the other caspases have two potential aspartate cleavage sites, resulting in removal of a linker region. Degrees of inhibition by cowpox viral serpin CrmA: + + +, potent inhibition; \pm , very weak inhibition; ?, not known.

Caspase	Other names	Active site	Cleavage site(s) between large and small subunits	CrmA inhibition
Caspase-1	ICE	QACRG	WFKD ↓ S; FEDD ↓ A	+ + +
Caspase-2	Nedd2, ICH-1	QACRG	DQDD ↓ G; EESD ↓ A	\pm
Caspase-3	CPP32, Yama, apopain	QACRG	IETD ↓ S	\pm
Caspase-4	ICE _{NI} , TX, ICH-2	QACRG	WRVD ↓ S; LEED ↓ A	+ + +
Caspase-5	ICE _{NI} , TY	QACRG	WRVD ↓ S; LEAD ↓ S	?
Caspase-6	Mch2	QACRG	DVVD ↓ N; TEVD ↓ A	\pm
Caspase-7	Mch3, ICE-LAP3, CMH-1	QACRG	IQAD ↓ S	\pm
Caspase-8	MACH, FLICE, Mch5	OACQG	VETD ↓ S; LEMD ↓ L	+ + +
Caspase-9	ICE-LAP6, Mch6	OACGG	DQLD ↓ A	?
Caspase-10	Mch4	OACQG	SQTD ↓ V; IEAD ↓ A	\pm

ICE-like proteases in apoptosis is the ability of specific protease inhibitors, including the cowpox viral serpin CrmA [21–23] and baculovirus p35 [24], to inhibit apoptosis (see below).

Since the recognition of the similarity between CED-3 and ICE in 1993, a further nine related ICE-like proteases have been identified. Several reviews have appeared on ICE and ICE-related proteases [7,15,16,25–29]. Due to the flurry of activity to isolate new family members, confusion has arisen as a result of different groups isolating the same protease. In order to resolve this, a unified nomenclature has recently been suggested [30] (Table 1). The trivial name proposed for all family members is caspase, the 'c' denoting a cysteine protease and the 'aspase' referring to the ability of these enzymes to cleave after an aspartic acid residue. Individual family members are then referred to in order of their publication, so ICE, the first family member, is caspase-1. Caspases are synthesized as inactive proenzymes, which are activated following cleavage at specific aspartate cleavage sites. Phylogenetic analysis of the caspases reveals that there are three subfamilies: an ICE subfamily, comprising

caspases-1, -4 and -5, a CED-3/CPP32 (32 kDa cysteine protease) subfamily, comprising caspases-3, -6, -7, -8, -9 and -10, and an ICH-1 (where ICH is *Ice* and *ced-3* homologue)/Nedd2 subfamily (Figure 2a and Table 2).

The presence of a family of structurally related caspases in cells raises a number of important questions in relation to their potential roles in cell death. (1) Are all caspases required for cell death, or are some members more important than others? (2) Do all modes of cell death utilize the same caspases? (3) Are caspases activated in series or in parallel? (4) Is one caspase at the apex of an apoptotic cascade? (5) How are these enzymes regulated in order to prevent their unwanted activation and the subsequent demise of the cell? (6) Are there normal cellular substrates for these enzymes? (7) What are their critical cellular substrates that lead to cell death? (8) Do specific caspases degrade specific proteolytic substrates during the apoptotic process? (9) Are the caspases pre-existing or are they synthesized in response to apoptotic stimuli? (10) Are the caspases tissue-specific? In this review, individual caspases will be discussed, paying particular attention to ICE, as most is known about this enzyme, and a detailed consideration of its properties is extremely helpful in understanding those of other family members. Then some of the known protein substrates of the caspases and their relationship with apoptosis will be reviewed.

ICE (CASPASE-1)

Structure and function

Early work on caspase-1 concentrated on its role in cleaving the inactive 31 kDa cytokine pro-IL-1 β at Asp-116-Ala-117 to generate the active 17 kDa mature form of IL-1 β , a key mediator of inflammation [31,32]. Purification and cloning of caspase-1 revealed that it is a 45 kDa protein (p45). Active ICE comprises two subunits of 20 kDa and 10 kDa (p20 and p10 respectively), both of which are required for catalytic activity and are derived from a single proenzyme following removal of an 11 kDa N-terminal peptide (prodomain) and a 2 kDa linker peptide (Figure 3) [33,34]. This scheme serves as a useful model for other caspases, although not all possess linker regions and the sizes of the prodomains vary (Figure 3). All four cleavage sites in p45, i.e. Asp-103, Asp-119, Asp-297 and Asp-316, arise at Asp-Xaa bonds (Figure 3), suggesting that active caspase-1 may be derived by autoproteolysis [33,34]. Following the initial cleavage at Asp-297-Ser-298, autoproteolysis occurs in a series of steps, generating fragments of increasing activity and eventually producing p20/p10 ICE [35,36]. Caspase-1 is found predominantly in the cytoplasm of cells as the p45 pro-form [37], although some is also localized to the external cell surface membrane, where it activates pro-IL-1 β to its mature form during secretion [38]. Caspase-1 is a novel type of cysteine protease containing an active-site cysteine residue (Cys-285) in the p20 subunit, the mutation of which results in loss of activity. Substrate specificity studies revealed that caspase-1 has a strong preference for aspartic acid adjacent to the cleavage site in the P₁ position, a small hydrophobic amino acid (Gly or Ala) in the P'₁ position, and also a requirement for four amino acids to the left of the cleavage site [33,39]. Acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO, where CHO is aldehyde) and Ac-YVAD-7-amino-4-methylcoumarin (Ac-YVAD-AMC) were synthesized as a potent competitive reversible inhibitor and a fluorimetric substrate respectively for caspase-1 [33].

The X-ray crystal structure of caspase-1, in complex with specific tetrapeptide inhibitors that bind in the S₁-S₄ sites normally occupied by a peptide substrate, has been determined. The active enzyme is a tetramer of two p20 subunits surrounding

two adjacent p10 subunits, with most of the area of contact between the dimers occurring between the p10 subunits [40,41]. Interactions between the p20 C-terminus and the p10 N-terminus also contribute to the stability of the homodimer. The active site spans both the p20 and p10 subunits, so explaining the requirement for both subunits for activity. Cys-285 and His-237 form a catalytic dyad in the active site of caspase-1. The active-site pentapeptide, Gln-Ala-Cys-Arg-Gly (QACRG), is in the p20 subunit. However, amino acid residues involved in forming the Asp pocket include Arg-179, Gln-283, Arg-341 and Ser-347, with only the first two residues being contributed by the p20 subunit. The two arginine residues (Arg-179 and Arg-341) form hydrogen bonds with the P₁ Asp residue of the substrate, and mutation of these residues results in the loss of catalytic activity [41]. Side chains of residues of p10 from Val-338 to Pro-343 interact with P₂-P₄ sites of the inhibitor [41]. Based on the crystal structure, two models were proposed for maturation of the proenzyme. In the first, two precursor p45 proteins associate and are then processed, with the p10 subunit from one caspase-1 molecule complexing with the p20 subunit from another caspase-1 molecule, so creating the active site. The alternative, but less favoured, model suggested processing followed by association of the subunits [41]. Using various active-site mutants, it has been shown that oligomerization of caspase-1 is required for autoproteolysis [42] and that hetero-oligomerization may occur between different caspase homologues, but the *in vivo* relevance of this is not known. Using the yeast two-hybrid system, the prodomain of caspase-1 is absolutely required for dimerization and autoproteolysis, suggesting a regulatory rather than a structural role for the prodomain [43]. Using reverse transcriptase-PCR, four alternatively spliced isoforms of caspase-1 were identified that have differing effects on apoptosis [44].

Caspase-1 and apoptosis

Early studies pointed to a role for caspase-1 in the induction of apoptosis. CED-3 and caspase-1 share 28% sequence identity, and the active-site pentapeptide, QACRG, is completely conserved (Table 2) [20]. Overexpression of the murine ICE gene induces apoptosis in Rat-1 fibroblasts, which is abrogated by point mutations in the cysteine or glycine residues of the active-site pentapeptide and by either *bcl-2* or *crmA* [21]. The *crmA* gene (a cytokine response modifier gene) encodes a 38 kDa serpin that is a specific inhibitor of caspase-1, so preventing the proteolytic processing of IL-1 β and thereby helping to suppress the response to viral infection [45]. As apoptosis is a major mechanism by which a host attempts to clear virally infected cells, viruses have evolved proteins that inactivate apoptosis in order to infect the host. Dorsal root ganglion neurons, which undergo apoptosis on withdrawal of nerve growth factor, are also protected by *crmA*, suggesting that caspase-1 may be involved in neuronal death in vertebrates [22].

Mice deficient in caspase-1 develop normally, appear healthy and are fertile, with no apparent abnormalities, suggesting that there are no gross defects in normal physiological processes involving apoptosis [46,47]. Macrophages from caspase-1^{-/-} mice are equally susceptible to ATP-induced apoptosis as those from wild-type mice. Thymocytes from caspase-1^{-/-} mice and wild-type mice show a similar susceptibility to apoptosis induced by dexamethasone or γ -irradiation, but thymocytes from the caspase-1^{-/-} mice are somewhat more resistant to apoptosis induced by anti-CD95 (Fas/APO-1) antibody [46,47]. These results suggest that caspase-1 in itself is not involved in most forms of apoptosis, or that another caspase may substitute in the

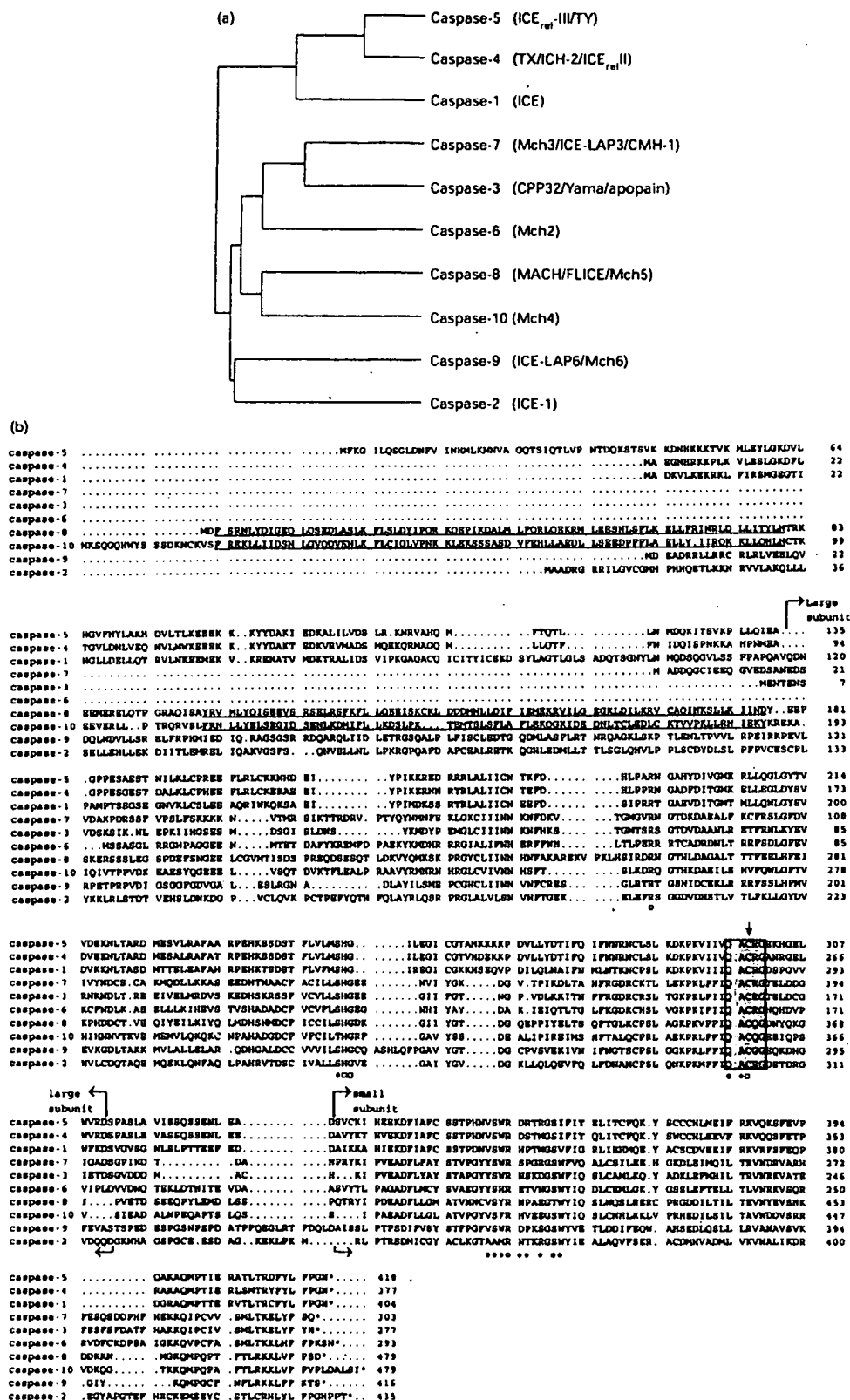


Table 2 Sequence identity of the caspases

The sequence identity between the full-length caspases shown in Figure 2(b) was analysed using the GAP program from the Genetics Computer Group.

Caspase	Identity (%)									
	1	2	3	4	5	6	7	8	9	10
1	100	22	30	55	50	22	26	22	25	22
2		100	22	27	22	28	22	26	33	28
3			100	33	30	33	52	33	37	33
4				100	77	28	22	20	22	21
5					100	22	25	22	24	22
6						100	33	35	33	35
7							100	33	32	33
8								100	22	41
9									100	33
10										100

caspase-1^{-/-} mice. The only exception is the suggestion of a role for caspase-1 in CD95-induced apoptosis in thymocytes. However, no autoimmune pathologies are seen in the caspase-1^{-/-} mice similar to those caused by the *lpr/lpr* mutation in the murine locus [48].

Further support for a role for caspase-1 in CD95-induced cell death was provided by the findings of a decrease in CD95-induced death due to CrmA, caspase-1 inhibitory peptides and caspase-1-specific antisense oligonucleotides [49,50], together with a small transient increase in a caspase-1-like activity prior to an increase in a CPP32 (caspase-3)-like proteolytic activity [51]. However, this elevation in a caspase-1-like activity has not been demonstrated to be essential for CD95-mediated apoptosis [51]. A role for caspase-1 has also been proposed in the apoptosis of mammary epithelial cells following loss of the extracellular matrix [52] and in DNA-damage-induced interferon regulatory factor-1-dependent T-lymphocyte apoptosis [53]. Similarly, caspase-1 is activated by growth factor deprivation, and suppression of this activation by growth factors such as insulin-like growth factor-1 and insulin also inhibits cell death [54]. In contrast, caspase-1 activity and apoptosis are uncoupled in macrophages undergoing apoptosis [55]. While there is some experimental support for a role for caspase-1 in apoptosis, in particular in CD95-mediated apoptosis, most data (see also below) suggest that other caspases may be of greater significance than caspase-1.

ICH-1/Nedd2 (CASPASE-2)

Nedd2 was originally identified using subtraction cloning as a developmentally down-regulated gene in mouse brain [56,57]. Using a murine *Nedd2* cDNA, a human foetal brain cDNA library was screened at low stringency, and *Ich-1*, the human homologue of *Nedd2*, was identified [58]. Both *Nedd2* and *Ich-1* encode proteins similar to caspase-1, and sequence alignment shows conservation of many important residues, including the

active-site pentapeptide QACRG (Table 1 and Figure 2) [57,58]. The *Ich-1* mRNA is alternatively spliced into two forms, one encoding a protein of 435 amino acids (ICH-1_L) and the other encoding a protein of 312 amino acids (ICH-1_S); a truncated form of ICH-1_L [58]. Overexpression of *Ich-1_L* in some, but not all, cell types results in apoptosis, whereas overexpression of *Ich-1_S* suppresses apoptosis induced by serum withdrawal, suggesting that *Ich-1* may play a role in both the positive and negative regulation of programmed cell death [58]. The enzymic activities of ICH-1_L/Nedd2 are required to cause cell death, as overexpression of mutant *Ich-1_L*/Nedd2 (with a Ser or Gly respectively replacing the active-site Cys) results in loss of activity [57,58]. During embryonic development, *Nedd2* is expressed at relatively high levels in various tissues, including the central nervous system, liver, kidneys and lungs [57]. Both the kidney and central nervous system are associated with high levels of programmed cell death during development [59]. *Nedd2* is also expressed to varying extents in several adult tissues, including post-mitotic neurons [57].

Two recent studies have addressed the question of which Asp cleavage sites are important in the processing of Nedd2 [60,61]. The Asp-333 → Ala mutant lacks apoptotic activity and does not produce p20 and p10 fragments, suggesting it as the cleavage site at the C-terminus of p20. Asp-347 was identified as the Asp residue at the N-terminus of p10. Asp-135 appears to be a cleavage site upstream of p20, as cell death activity and processing are prevented when it is mutated [60,61]. The proposed cleavage sites in Nedd2 are conserved in caspase-2 (Figure 3). Caspase-2 may be activated *in vitro* by caspase-1, caspase-3 and granzyme B [61]. Recently, the cleavage of caspase-2 to its catalytically active subunits during the execution phase of apoptosis in the human monocytic tumour cell line THP.1 was demonstrated [62]. Caspase-2 was cleaved early during the apoptotic process, but it was not possible to discern whether its cleavage preceded that of other caspases. As yet, no specific intracellular protein substrates of caspase-2 have been identified.

CPP32/YAMA/APOPAIN (CASPASE-3)

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which are cleaved in many different systems during apoptosis (see below). Using the DNA sequence encoding the active site of caspase-1 and CED-3 to search an expressed sequence tag database, a human sequence was identified, cloned and shown to encode a 32 kDa cysteine protease, called CPP32 [63]. Independently, two other groups identified caspase-3, one naming it Yama (the Hindu god of death) and the other apopain [64,65]. Caspase-3, a member of the CED-3 subfamily of caspases (Table 2 and Figure 2b), is widely distributed, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system [63]. Based on the known cleavage site of PARP (DEVD₁CG), Ac-DEVD-AMC was

Figure 2 (a) Phylogenetic relationships and (b) sequence alignment of the caspases

Phylogenetic relationships were determined and polypeptide sequences for the human forms of the caspases aligned using the PILEUP algorithm (Genetics Computer Group, Madison, WI, U.S.A.). The relationships are based on the full-length proenzymes, which is appropriate as the prodomains clearly play a functional role. The dendrogram is not affected if the same analysis is carried out using caspases without their prodomains. Amino acid residues are numbered to the right of each sequence. The QACXG motif is conserved in all family members and is boxed. A coloured arrow above the aligned sequences indicates the position of the catalytic cysteine residue. Amino acids that align with residues within ICE that have been shown by the X-ray crystal structure to be involved in binding P₁ Asp (○), in catalysis (□) and adjacent to substrate P₂-P₄ amino acids (◆) are indicated. The underlined sequences represent the FADD/MORT1 (see the text) homology domains in caspase-8 and caspase-10.

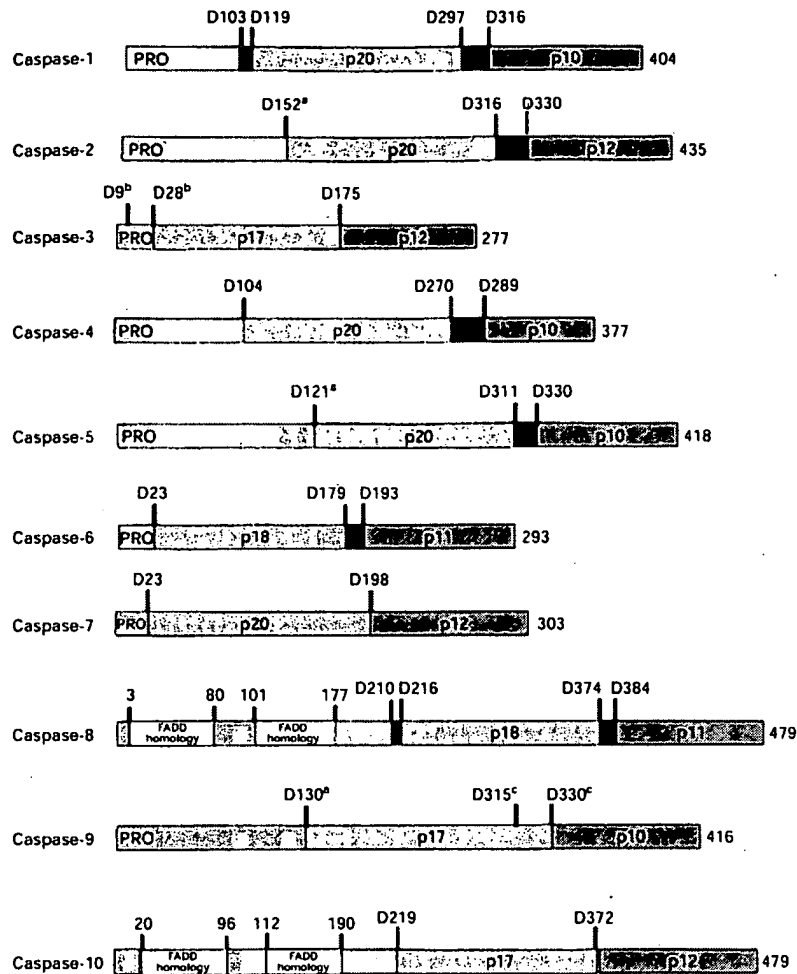


Figure 3 Proenzyme organization of the caspases

Caspases are synthesized as proenzymes, with a N-terminal peptide or prodomain (PRO), and two subunits sometimes separated by a linker peptide (black box). Based on caspase-1 and caspase-3, active enzymes are heterotetramers of two large (~ 20 kDa; p20) and two small (~ 10 kDa; p10) subunits. The proenzymes are cleaved at specific Asp residues (D_n, where *n* is the position in the protein). The numbers at the right-hand side are the numbers of amino acids in the protein. *Exact cleavage site not known; ^bthe cleavage site of caspase-3 may be at Asp-9 or Asp-28 [65–67]; ^ccaspase-9 is cleaved preferentially at Asp-330 by caspase-3 and at Asp-315 by granzyme B [82]. Caspase-2 cleavage sites are based on equivalent sites being present in Nedd2 [60,61]. FADD represents the domains of caspase-8 and caspase-10 that are homologous to the DED of FADD/MORT1.

synthesized as a model substrate, and Ac-DEVD.CHO and its biotinylated derivatives were synthesized as specific inhibitors of PARP cleavage and as affinity ligands for purification of the protease. Using electrospray MS and N-terminal sequence analysis, the active enzyme was shown to be composed of two subunits of 17 kDa and 12 kDa, derived from the precursor protein by cleavage at Asp-28–Ser-29 and Asp-175–Ser-176 [65] (Figure 3). While the initial cleavage is probably between the large and small subunits, it has been suggested that processing within the prodomain occurs initially at Asp-9, not at Asp-28 [66,67].

During the execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contains a common Asp-Xaa-Xaa-Asp (DXXD) motif (Table 3), similar to that originally described in PARP [68]. In comparison with caspase-1, caspase-

3 has no linker peptide and the prodomain is much shorter (Figures 2b and 3). Caspase-3 prefers a DXXD-like substrate, whereas caspase-1 prefers a YVAD-like substrate [65]. Both enzymes have an almost absolute requirement for an Asp in the P₁ position and both can tolerate a fair degree of substitution in the P₂ and P₃ positions, but in the P₄ position caspase-1 prefers a hydrophobic amino acid such as Tyr, whereas caspase-3 has a marked preference for an Asp. The three-dimensional structure of a complex of caspase-3 with DEVD.CHO, a potent tetrapeptide aldehyde inhibitor, shows that, although caspase-3 resembles caspase-1 in overall structure, its S₄ subsite is very different in size and chemical composition and accounts for their differences in specificity [69]. The S₄ subsite of caspase-1 is a large shallow hydrophobic depression that readily accommodates a tyrosyl side chain, while the analogous site in caspase-3 is a narrow pocket that closely surrounds the P₄ Asp side chain [69].

Table 3 Protein substrates of caspases

Abbreviation: SREBP, sterol regulatory element binding protein.

Protein substrate	Cleavage motif	Caspase(s)	Function of substrate	References
PARP	DEVD ↓ G	3,7	DNA repair enzyme	68, 104
U1-70 kDa	DGPD ↓ G	3	Splicing of RNA	106
DNA-PK _{cs}	DEVD ↓ N	3	DNA double-strand-break repair	106, 107
Gas2	SRVD ↓ G	?	Component of microfilament system	126
Protein kinase C δ	DMQD ↓ N	3	Cleaved to active form in apoptosis	130, 131
Pro-IL-1 β	YVHD ↓ A	1	Cleaved to mature active cytokine	32–34
D4-GDP dissociation inhibitor	DELD ↓ S	3	Regulator of Rho GTPases	110
Lamin A	VEID ↓ N	6	Assist in maintaining nuclear shape	113–116
Heteroribonuclear proteins C1 and C2	?	3,7	Processing of pre-mRNA	108
Huntingtin	DXXD	3	Huntington disease gene product	111
SREBP-1 and SREBP-2	DEPD ↓ S	3,7	Sterol regulatory element binding proteins	109
Fodrin	DETD ↓ S?	?	Membrane-associated cytoskeletal protein	127–129
Rb (see the text)	DEAD ↓ G	3	Cell cycle regulatory protein	112, 133, 134

The Trp residue at position 348 and an inserted 10-amino-acid sequence at position 381 (for ease of comparison, the residue numbers used are those of the analogous residues in caspase-1) play a crucial role in defining the size and shape of the S₁ subsite of caspase-3 and are also conserved in all known members of the CED-3 subfamily (Figure 2b). The activation of caspase-3 to either of its catalytically active p17 or p12 subunits has been demonstrated in cells undergoing apoptosis [70–72].

Caspase-3-deficient mice, generated by homologous recombination, are smaller than their littermates and die at 1–3 weeks of age. Thymocytes from caspase-3-deficient mice show a similar sensitivity to apoptosis induced by a number of different stimuli, including CD95, anti-CD3, staurosporine and dexamethasone. Brain development in these deficient mice is markedly affected, with a variety of hyperplasias being observed from embryonic day 12. Pyknotic clusters of apoptotic cells, observed at sites of major morphogenetic change in normal brain development, are not seen in the deficient mice, indicative of decreased apoptosis in the absence of caspase-3. This demonstrates that caspase-3 plays a critical role during morphogenetic cell death in the mammalian brain and also that mutation of a mammalian homologue of *Ced-3* leads to decreased cell death and a supernumerary cell population during development, emphasizing that the basic cell death machinery is evolutionarily conserved. The restricted phenotype also raises the possibility that other caspases may be important in other tissues or cell types [73].

Cytotoxic T lymphocytes kill target cells containing foreign antigens by either CD95- or granule-mediated cytotoxicity. Exocytosis of cytotoxic T lymphocyte granules allows perforin to polymerize in the target cell membrane, so facilitating entry of the granzymes, a family of serine proteases. Granzyme B also exhibits an unusual specificity for Asp in the P₁ position. *In vitro*, granzyme B can activate caspase-3, as assessed by formation of its p17 subunit as well as its ability to cleave PARP to its signature fragment [74,75]. Activation of caspase-3 by granzyme B is within the physiological range [75]. While granzyme B can activate directly caspase-3, it may also cleave another caspase which in turn activates caspase-3.

ICE_{re}II/TX/ICH-2 (CASPASE-4) AND ICE_{re}III/TY (CASPASE-5)

Three groups independently cloned ICE_{re}II/TX/ICH-2 (caspase-4) [76–78] and two groups cloned ICE_{re}III/TY (caspase-5) [76,79] (Figure 2b). Both caspase-4 and caspase-5 are

members of the caspase-1 subfamily, and are more closely related to each other than to other homologues (Table 2). Caspase-4 expression, while lower than that of caspase-1, generally shows a similar tissue distribution, being found in most tissues examined with the exception of brain. Appreciable levels are found in both lung and liver, and also in ovary and placenta, where caspase-1 mRNA is barely detectable [76,77]. Caspase-5 is expressed at a much lower level than caspase-4 [76,79]. Caspase-4 and caspase-5 have different substrate specificities from that of caspase-1, being much poorer at cleaving pro-IL-1 β [76–79]. Caspase-4 may be involved in the maturation of caspase-1 [78]. At high concentrations, caspase-4 cleaves PARP [80], but the biological relevance of this is unclear. Limited information is available concerning the precise sites at which caspase-4 and caspase-5 are processed. Maturation of caspase-1 results from cleavage after Asp residues at positions 119, 297 and 316, with the latter two residues being conserved in caspase-4 (Asp-270 and Asp-289) and caspase-5 (Asp-311 and Asp-330); by analogy, this probably represents the removal of a linker peptide (Table 1 and Figure 3). The Asp-119 site in caspase-1 is not conserved in caspase-4, but Asp-104 might define the cleavage site for removal of the prodomain [77]. Overexpression of caspase-4 and caspase-5 generally results in apoptosis, although in some studies apoptosis was only induced after removal of the prodomains, suggesting that the prodomains may be involved in the regulation of apoptosis [76,78].

Mch2 (CASPASE-6)

Using a PCR approach with degenerate primers encoding two highly conserved pentapeptides, QACRG and GSWFI, Alnemri and co-workers cloned Mch2 (a mammalian CED-3 homologue) from a human Jurkat T lymphocyte cDNA library [81]. Two transcripts were detected, Mch2 α (1.7 kb) and Mch2 β (1.4 kb), the former encoding the full-length Mch2 and the latter encoding a shorter isoform, possibly as a result of alternative splicing. Mch2 α (caspase-6) encodes a 293-amino-acid protein with a predicted molecular mass of ~34 kDa. Mch2 β contains a deletion corresponding to nucleotides 119–385 of the Mch2 α sequence (amino acids 14–102) and encodes a 204-amino-acid protein with a predicted molecular mass of ~23 kDa. Mch2 β lacks half of the p20 subunit and is probably catalytically inactive [81]. Expression of Mch2 α , but not Mch2 β , in insect cells results in the induction of apoptosis. Caspase-6 is a member of

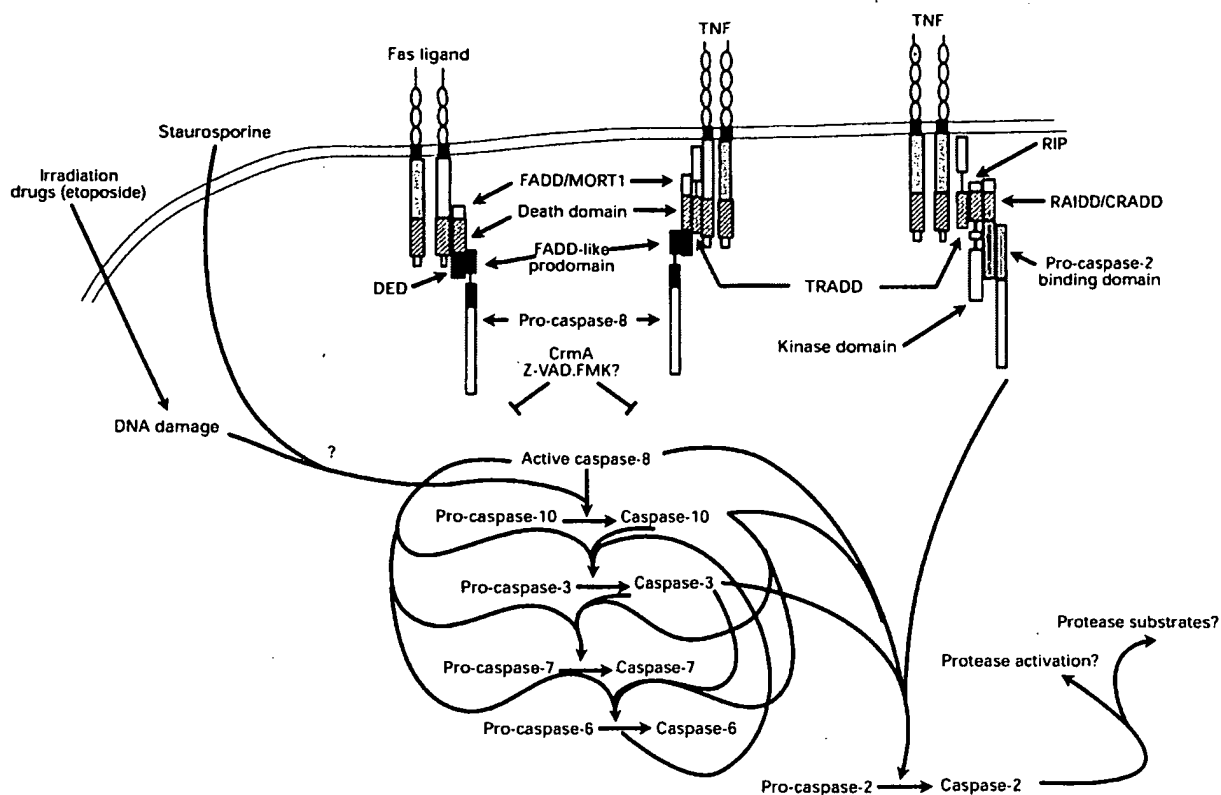


Figure 4 Hypothetical hierarchy of caspases

Apoptosis can be triggered by drugs (staurosporine), by DNA-damaging agents (etoposide or γ -irradiation) or by CD95 (Fas/APO-1) or tumour necrosis factor (TNF) interacting with their respective receptors. Both of these receptors transduce their apoptotic signals via intracellular C-terminal death domains (hatched boxes) which are involved in protein-protein interactions. Both CD95 and TNF result in the cleavage and activation of caspase-8, which can cleave all other known caspases. Caspase-10, which also cleaves all other known caspases, is activated by different stimuli via a CrmA-insensitive pathway, and may be activated following DNA damage (?). Activation of some caspases, such as caspase-6, results in their being able to cleave the caspase initially responsible for their formation, so setting up a protease amplification cycle [82]. Caspases may also be recruited to the death pathways by adapter molecules such as RAIDD/CRADD, which bind to RIP and to the prodomains of certain caspases such as pro-caspase-2 [101,102]. Part of this scheme, showing which caspase activates other caspases, is based on *in vitro* studies with purified enzymes, and requires confirmation from studies with intact cellular systems.

the CED-3 subfamily, showing high identity with caspase-3 (Figure 2a and Table 2).

Caspase-6, when expressed as a glutathione S-transferase fusion protein in *Escherichia coli*, autoprocesses and cleaves Ac-DEVD-AMC [although it is much less active (~150-fold) than caspase-3], but it does not cleave Ac-YVAD-AMC. Caspase-6 is also not readily inhibitable by Ac-DEVD-CHO [82]. Recombinant caspase-6 cleaves human PARP to give a smaller fragment than that seen in cells undergoing apoptosis, suggesting that caspase-6 does not play a major role in the cleavage of PARP [81]. Purified human recombinant caspase-3 cleaves 35 S-labelled pro-caspase-6 at three aspartate cleavage sites (Asp-23, Asp-179 and Asp-193), resulting in the formation of the large (p18) and small (p11) subunits of caspase-6 [82]. Initial cleavage appears to be at D¹⁷⁹VVDI¹⁸⁰, resulting in a p13 subunit, which is then further cleaved at T¹⁹³EVDI¹⁹⁴, resulting in the p11 subunit (Figure 3). Activation of pro-caspase-6 by caspase-3 results in an active enzyme that is capable of cleaving an artificially introduced lamin cleavage site (VEID¹N) [82]. These data suggest that caspase-3 is activated prior to, and may be responsible for, the activation of caspase-6. However, these results are in apparent

conflict with a recent study which identified and purified from hamster liver a homologue of caspase-6 that is capable of activating caspase-3 [83]. In addition, caspase-6 processes pro-caspase-3 at the I¹⁷²ETD¹S¹⁷⁴ site between the large and small subunits, and this cleavage is blocked when Asp-175 is mutated to Ala [82]. Thus activation of caspase-3 can result in activation of pro-caspase-6 but, similarly, activation of pro-caspase-6 can also result in activation of caspase-3, resulting in a protease amplification cycle (Figure 4) [82]. Some caution must be exercised in the interpretation of such *in vitro* experiments, as purified or partially purified proteases may cleave substrates that they would not cleave *in vivo*, either because the ratio of enzyme to substrate would never reach such a level in the cell or because the enzyme may be located in a different subcellular environment from the substrate.

Mch3/ICE-LAP3/CMH-1 (CASPASE-7)

Caspase-7 was cloned independently in three different laboratories and named Mch3/ICE-LAP3/CMH-1 [84-86]. A member of the CED-3 subfamily, it is a 303-amino-acid protein with high

similarity to caspase-3 (Figure 2a and Table 2). An alternatively spliced isoform of caspase-7, which may act as a negative regulator of apoptosis, has been described [84]. Caspase-7 is constitutively expressed in many foetal and adult tissues, with lowest expression observed in the brain. Using a rabbit anti-peptide antibody, caspase-7 protein migrates at ~35 kDa and is detected to a variable extent in a number of cell lines, including Jurkat T cells, where it is localized diffusely to the cytoplasm and juxtamembrane structures [85], consistent with the suggestion that the death effector machinery resides in the cytoplasm rather than the nucleus [8]. Overexpression of full-length caspase-7 in the MCF7 breast carcinoma cell line does not induce apoptosis, whereas expression of a truncated derivative, lacking the 53 N-terminal amino acids corresponding to the putative prodomain, induces apoptotic cell death [85]. Bacterially expressed caspase-7, like caspase-3, preferentially cleaves PARP and the peptide substrate Ac-DEVD-AMC, but not Ac-YVAD-AMC or pro-IL-1 β . The competitive peptide aldehyde inhibitor Ac-DEVD-CHO is a potent inhibitor of both caspase-3 and caspase-7, whereas Ac-YVAD-CHO and CrmA are poor inhibitors of both of these enzymes. As caspase-3 and caspase-7 are functionally similar and have similar substrate specificities [84], cleavage of PARP during apoptosis may be due to a combination of the action of both these caspases. Active caspase-7 is made up of two subunits, similar to other caspases [66] (Figure 3). Caspase-7 is activated to its catalytically active large subunit in intact cells undergoing apoptosis [62,85,87].

Following cleavage at Asp-198 and Asp-23, granzyme B activates pro-caspase-7 to a form that cleaves PARP to its signature fragment of ~85 kDa [66,88,89]. Using a caspase-7 protein with Asp-23 mutated to glutamate, it was demonstrated that removal of the prodomain by autoproteolysis is not necessary for activity and that the p25/p12 enzyme is as active as the p20/p12 enzyme [89]. This suggested that the prodomain of caspase-7 may be involved in an as yet uncharacterized way in the regulation of caspase-7. *In vitro*, caspase-7 is a better substrate for granzyme B than is caspase-3. Pro-caspase-7 appears to exist as dimers or higher-order oligomers [89]. Incubation of Jurkat T cells with granzyme B together with a sublytic concentration of perforin results in the activation of endogenous caspase-7 prior to the induction of apoptosis. Together, these data suggest that caspase-7 may be an important intracellular effector of granzyme B-mediated apoptosis and cytotoxic T-lymphocyte-induced cell killing *in vivo*.

MACH/FLICE/Mch5 (CASPASE-8)

CD95 and the TNF receptor (TNFR-1; p55-R) are members of the TNF/nerve growth factor receptor family. Activation of these cell-surface cytokine receptors, either by their natural ligands or by agonistic antibodies, results in apoptosis. CD95 and TNFR-1 share a region of identity termed the 'death domain' that is required to signal apoptosis. Using a yeast two-hybrid system, three proteins were identified that bind to either the intracellular domains of CD95 and/or TNFR-1 through hetero-association of homologous regions found in these proteins. FADD/MORT1 (Fas-associating protein with death domain) [90,91] binds specifically to CD95, TRADD (TNFR-1-associated death domain protein) binds to TNFR-1 [92], and RIP (receptor-interacting protein) binds to both receptors [93]. Activation of CD95 initiates the association of at least four proteins (CAP1-CAP4), two of which (CAP1 and CAP2) have been identified as alternative forms of phosphorylated FADD/MORT1 [94]. A dominant-negative version of FADD, lacking the N-terminal death effector domain (DED), blocks the recruit-

ment of the two other proteins (CAP3 and CAP4) to the death-inducing signalling complex [91], suggesting that these two proteins are downstream components of the CD95 signalling cascade. The region encompassing the 117 N-terminal amino acid residues of FADD can trigger apoptosis, and have been called DED [95].

Two groups independently identified a novel caspase, named MACH/FLICE (caspase-8), which contains both an active subunit with identity with the caspases and an N-terminal prodomain containing two domains with marked identity with the N-terminal DED of FADD/MORT1 [96,97] (Figure 3). Using nano-electrospray tandem MS, peptide sequences were obtained for CAP4 which, when used as sequence tags, identified a 3 kb cDNA that encodes a novel protein, caspase-8, of predicted molecular mass 55.3 kDa. The other group used a yeast two-hybrid screen to identify a cDNA clone with a novel sequence which binds to MORT1/FADD. This novel protein (MACH) occurs in multiple isoforms, most probably produced by alternative splicing, some of which contain a region with identity with the caspases. Northern blot analysis revealed a heterogeneity of caspase-8 transcripts which varied in amount and size in different human tissues. Few caspase-8 transcripts are detectable in testis, skeletal muscle and brain, with a relatively higher level of expression in peripheral blood leucocytes, consistent with a role for CD95-induced apoptosis in lymphocyte homeostasis.

Caspase-8 contains two N-terminal stretches of approx. 70 amino acids that are apparently homologous to the DED of FADD. Residues 7-75 and 101-169 of caspase-8 share 39% identity (55% similarity) and 28% identity (55% similarity) respectively with the DED of FADD (residues 4-76) [97]. While the N-terminal portion of caspase-8 contains the FADD homology domains, the remainder of the protein is highly similar to the CED-3 subfamily of caspases (Figure 2a and Table 2). Instead of the active-site QACRG pentapeptide found in most caspases, caspase-8 contains the novel sequence QACQG. Caspase-8 associates with the DED of FADD. Granzyme B activates caspase-8 to an active protease which cleaves PARP to its characteristic signature fragment. Overexpression of caspase-8 results in apoptosis, and mutation of its catalytic cysteine residue abolishes its apoptotic potential. Expression of caspase-8 in the presence of isoforms with an incomplete caspase region resulted in little cell death. Similarly, these isoforms lacking a complete caspase region blocked cell death induced by CD95 and TNF, suggesting that the isoforms exert a dominant-negative effect and may be important in the regulation of apoptosis *in vivo*.

The marked heterogeneity of isoforms of caspase-8 compared with other caspases may provide a mechanism for some tissues or cells to protect themselves against CD95- or TNF-induced cell death [96]. These studies provide a critical link between activators and effectors of the cell death machinery. Oligomerization of the death domain of either CD95 or TNFR-1 allows recruitment of cytosolic adapter proteins to assemble a death-inducing signalling complex [98]. Thus CD95 (a cell death receptor) uses FADD (an adapter molecule) to interact physically with caspase-8 (a cytosolic protease) and initiate the apoptotic cascade (Figure 4). The precise mechanism by which recruitment of caspase-8 results in its activation is not known. It has been suggested that, in the latent state, the two DEDs of caspase-8 bind to each other, so preventing activation. The binding of FADD following the triggering of apoptosis by CD95 or TNF may cause a conformational change in the DED of FADD, so facilitating its binding to one of the DEDs of caspase-8, thereby disrupting the association of the two DEDs of caspase-8 and allowing its caspase domain to undergo autocatalytic activation [96,97].

Independently, Mch5 was cloned from Jurkat T cells, and its predicted sequence is almost identical with that of caspase-8 [66,96]. A small difference in the prodomain is revealed following sequence comparison of Mch5 and MACH/FLICE (G. M. Cohen, unpublished work). Bacterial expression of pro-caspase-8 generates a mature enzyme composed of two subunits, derived by processing of the proenzyme (Figure 3). Recombinant caspase-8 is able to process/activate all known caspases, including caspases-1 to -7 and caspases-9 and -10 [66,67], supporting the suggestion that it lies at the apex of an apoptotic cascade, at least from some stimuli such as CD95 or TNF [96,97].

ICE-LAP6/Mch6 (CASPASE-9)

Recently, two groups independently cloned a new member of the caspase family, ICE-LAP6/Mch6 (caspase-9) [82,99]. On searching the databases for genes related to that for caspase-7, a cDNA clone was identified that encodes a novel 416-amino-acid protein with a predicted molecular mass of ~46 kDa. Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3 (Table 2). The major difference between caspase-9 and other family members is the active-site pentapeptide QACGG, in which a Gly is found instead of the usual Arg (Table 1). Pro-caspase-9 contains a long N-terminal putative prodomain with high similarity to the prodomains of CED-3 and caspase-2. Northern-blot analysis revealed the presence of multiple mRNA species, suggestive of alternative splicing. High levels of expression of caspase-9 are found in the heart, testis and ovary. Overexpression of caspase-9, but not of a mutant in which the catalytic Cys was replaced with an Ala, induced apoptosis in MCF7 cells.

Procaspase-9 contains two potential processing sites between its large and small subunits, P³¹²EPDJA³¹⁶ and D³²⁷QLDJA³³¹. The latter motif is similar to the DEVD↓G site in PARP, suggesting that caspase-9 may be activated by caspase-3, while the former motif may be a potential granzyme B cleavage site, as it contains an acidic residue in the P₃ position. Using *in vitro* mutagenesis, it was demonstrated that both caspase-3 and granzyme B activated pro-caspase-9, although to differently sized products. Asp-330 was the processing site for caspase-3, generating two products of molecular masses ~37 kDa (p37) and ~10 kDa (p10). Granzyme B cleaved procaspase-9 at both sites, with a marked preference for Asp-315 over Asp-330, generating an active enzyme capable of cleaving PARP to its signature fragment of ~85 kDa. In some, but not all, studies the prodomain of caspase-9 was removed [82,99]. The ability of caspase-3 to activate pro-caspase-9 suggests that the latter is downstream of caspase-3 and, as such, may be responsible for some of the later changes seen in cells undergoing apoptosis.

Mch4 (CASPASE-10)

Searching the databases of expressed sequence tags for sequences related to caspase-3 and caspase-6 led to the cloning from Jurkat T cells of a novel cDNA encoding a 479-amino-acid protein, Mch4 (caspase-10), with a molecular mass of ~55 kDa [66]. Caspase-10, a member of the CED-3 subfamily, is more closely related to caspase-8 than to any other caspase (Figure 2a and Table 2). Like caspase-8, caspase-10 has an active-site QACQG pentapeptide and also contains two FADD-like DEDs in its N-terminal domain, suggesting a possible role in CD95- or TNF-induced apoptosis. Mature caspase-10 is derived from a single-chain polypeptide proenzyme by cleavage at Asp-372 located between the large and small subunits (Figure 3). Northern blot analysis revealed that caspase-10 mRNA is present in most

tissues, with lowest expression being observed in brain, kidney, prostate, testis and colon and higher levels in heart, liver and spleen. Recombinant caspase-10 is unusual in that it has a similar K_m for the cleavage of both Ac-DEVD-AMC and Ac-YVAD-AMC, but it is more similar to caspase-3 as it is potently inhibited by Ac-DEVD-CHO [66]. Granzyme B cleaves procaspase-10, lacking the N-terminal FADD-like domains, at I³⁹⁹EADJA³⁷³. Purified recombinant caspase-10 processes all caspases, including pro-caspases-3, -7 and -10, whereas neither caspase-3 nor caspase-7 cleaves pro-caspase-10, suggesting that the latter is upstream of both caspase-3 and caspase-7 and lies at or near the apex of a cascade of proteases [66,67] (Figure 4).

GENERAL FEATURES OF THE CASPASES

Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (p20) and small (p10) subunits. In some cases these subunits are separated by a linker region of unknown function but which may be involved in regulation of the activation of the caspase. All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). The amino acids Cys-285 and His-237 involved in catalysis, and those involved in forming the P₁ carboxylate binding pocket in caspase-1 (Arg-179, Gln-283, Arg-341 and Ser-347), are conserved in all the other caspases, except for the conservative substitution of Thr for Ser-347 in caspase-8 (Figure 2b). This explains the absolute requirement for an Asp in the P₁ position. However, the residues that form the P₂-P₄ binding pocket are not well conserved, suggesting that they may determine the substrate specificities of the different caspases. It is evident from studies such as those with the caspase-1^{-/-} and caspase-3^{-/-} mice that not all caspases are required for cell death, and that some are more important than others. The importance of the tissue specificity of individual caspases is illustrated by the effects on the brains of caspase-3^{-/-} mice [73]. Alternatively spliced isoforms of many caspases may in part regulate the activity of the full-length enzyme, either by acting as dominant inhibitors or by forming inactive heteromeric complexes [58,96]. A key role for a particular caspase in apoptosis has often been inferred from its overexpression resulting in the induction of this process. However, such overexpression may lead to the caspase cleaving substrates that it does not normally recognize. In addition, injection of other proteases such as trypsin or chymotrypsin into the cytoplasm of various cell types results in the induction of apoptosis [100].

Some caspases contain only a short prodomain (caspases-3, -6 and -7), whereas others contain long prodomains (caspases-1, -2, -4, -5, -8, -9 and -10) (Figure 3). The importance of the FADD-like prodomains of caspase-8 and possibly caspase-10 in directly linking CD95- and TNFR-1-mediated apoptosis has already been emphasized [96,97]. The significance of the other prodomains is not known, but they may be important in regulation of the activation of the caspases [43]. This possibility has been highlighted by the identification of a new adapter molecule, RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain)/CRADD (caspase and RIP adapter with death domain) [101,102]. RAIDD/CRADD comprises two main domains: an N-terminal domain which resembles and binds by a homophilic mechanism to the prodomain of caspase-2 and CED-3, and a C-terminal death domain that binds to RIP, a Ser/Thr kinase, which associates with CD95 and induces death [101]. The N-terminus of RAIDD/CRADD has significant identity with the prodomains of caspase-2 (residues 15-91; 31% identity), caspase-9 (residues 1-79; 28% identity) and CED-3

(residues 2–78; 24% identity), as well as with the C-terminus of a human inhibitor of apoptosis (IAP-1) [102]. Mutation of highly conserved residues in the N-terminal prodomains of caspase-2 and CED-3 abolishes their binding to RAIDD. RAIDD binds RIP, part of the TNFR-1 signalling complex, but not FADD or TRADD, except in the presence of RIP, when it can bind the latter. RIP recruits RAIDD/CRADD, which in turn recruits caspase-2, so creating a direct link to the effector caspases [101,102] (Figure 4). Interestingly, mature caspase-2 processes its own precursor, but not other currently known caspases [102]. Thus prodomains of some caspases may enable them to be recruited specifically to facilitate the execution of the cell death programme. It remains to be determined whether there is a family of RAIDD-like molecules that can recruit other caspases with long prodomains.

PROTEIN SUBSTRATES CLEAVED BY CASPASES DURING THE EXECUTION PHASE OF APOPTOSIS

During the execution phase of apoptosis, several proteins, including PARP, lamin B and histone H1, are cleaved [103]. The number of proteins identified as being cleaved during apoptosis is increasing rapidly. The caspases responsible for these reactions are indicated in Table 3; however, other caspases may also cleave these substrates. It is also not known which caspase(s) is/are responsible for cleavage under physiological conditions, or whether there is redundancy in the proteases for these cleavages. Some caspases show overlapping specificities for some substrates (caspase-3 and caspase-7 can both cleave PARP), whereas other caspases may have a unique substrate specificity (to date, caspase-6 is the only caspase known to cleave lamins). The biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology is often not known. Caspase-3 is responsible, either wholly or in part, for the proteolytic cleavage of a large number of substrates during apoptosis, including PARP, DNA-dependent protein kinase (DNA-PK), U1-70 kDa, heteronuclear ribonucleoproteins C1 and C2, sterol regulatory binding proteins, D4-GDP dissociation inhibitor, huntingtin, and almost certainly retinoblastoma protein (Rb) [104–111] (Table 3). A common feature of all these substrates is the presence of a DXXD motif (Table 3), similar to that originally described in PARP [68]. Based on the cleavage site of PARP, Ac-DEVD-AMC was designed as a fluorimetric substrate for the measurement of caspase-3 activity. However, since caspase-7 also cleaves this substrate and this reaction is also potentially inhibited by Ac-DEVD-CHO, it is likely that many substrates described as being cleaved by caspase-3 will also be found to be cleaved by caspase-7.

PARP

PARP is possibly the best characterized proteolytic substrate of caspases, being cleaved in the execution phase of apoptosis in many systems, including thymocytes, HL-60 cells and breast cancer cell lines [103,104]. Intact PARP (116 kDa) is cleaved to 24 kDa and 89 kDa fragments, representing the N-terminal DNA binding domain and the C-terminal catalytic domain of the enzyme respectively [104]. This possibly conserves the cellular NAD⁺ and ATP normally required for PARP activity, thereby enabling the ATP to be utilized for the execution of apoptosis [7]. Cleavage of PARP may also interfere with its key homeostatic function as a DNA repair enzyme [105]. PARP is cleaved at the sequence DEVD|G by a protease activity resembling ICE (pRICE), but not by ICE itself [68]. Although the cleavage of

PARP is often a valuable indicator of apoptosis, its biological relevance, if any, is unclear, since PARP-null mice develop normally [112]. *In vitro*, other caspases, including caspases-2, -4, -6, -7, -8, -9 and -10, when added at high concentrations, can also cleave PARP or DEVD-AMC [80]. The physiological significance of this cleavage of PARP by these caspases is still under investigation. At the present time, it appears that caspase-3 and caspase-7 are primarily responsible for PARP cleavage during apoptosis.

DNA-PK

DNA-PK, an enzyme involved in DNA double-strand-break repair, possesses a 460 kDa catalytic subunit (DNA-PK_{cs}) and a DNA binding component Ku, which is a heterodimer of 70 and 80 kDa subunits. During apoptosis in several different systems, DNA-PK_{cs}, but not Ku, is degraded by a caspase with properties similar to those of caspase-3 [105,106]. Caspase-3, but not caspases-1, -4 or -6, cleaves purified DNA-PK_{cs} to fragments of a similar size to those observed in cells undergoing apoptosis. Degradation of DNA-PK_{cs} should lead to a decrease in the DNA repair capacity of the cell, so abolishing its key homeostatic function and facilitating the characteristic DNA degradation associated with apoptosis [105,106].

Lamins

The proteolysis of lamins, the major structural proteins of the nuclear envelope, is observed in different cells undergoing apoptosis [68,87,113–116] and may be responsible for some of the observed nuclear changes, since inhibitors of lamin cleavage prevent some of these changes [115,117]. An *in vitro* model of apoptosis has been developed in which normal nuclei, exposed to cytosol from apoptotic cells, undergo many of the characteristic biochemical and morphological changes of nuclear apoptosis, including chromatin condensation, fragmentation and margination, internucleosomal cleavage of DNA, and proteolysis of PARP and lamins [68,118]. In this model, the lamin protease is clearly distinct from the PARP protease, cleavage of PARP being significantly more rapid than that of lamins and less sensitive to inhibition by either YVAD chloromethyl ketone or tosyl-lysylchloromethane [115]. Caspase-6 (Mch2) cleaves lamin A at a conserved VEID|N sequence to give a fragment similar to that seen in apoptotic cells and extracts [119,120]. The site of cleavage is in a well conserved α -helical rod domain, which may disrupt lamin–lamin interactions as well as interactions of lamins with other nuclear components [120]. This lamin cleavage is readily inhibited by YVAD chloromethyl ketone. Under conditions where caspase-6 cleaves lamin A, caspases-1, -3 and -7 do not, suggesting that caspase-6 may be the major laminase in cells undergoing apoptosis [119,120]. Other, as yet untested, caspases may also cleave lamins, as may a Ca²⁺-regulated serine protease [115,121]. Pro-caspase-6 is activated in cells undergoing apoptosis induced either by anti-CD95 antibody or by staurosporine, and caspase-6 functions downstream of Bcl-2 and Bcl-x_L [119]. In CD95-treated HeLa cells, lamin B is preferentially cleaved early in apoptosis prior to cleavage of lamins A and C and internucleosomal cleavage of DNA; this suggests that B- and A-type lamins may be cleaved by different caspases [122].

U1-70 kDa

The activity of the U1 small nuclear ribonucleoprotein particle, which is essential for the splicing of precursor mRNA, is dependent on both the RNA and protein components, including U1-70 kDa. In several systems, including CD95- and TNFR-

induced apoptosis, U1-70 kDa is cleaved early in the apoptotic process to a 40 kDa fragment [105,123]. Purified caspase-3 cleaves U1-70 kDa at a DGPD[G] site, similar to the reaction observed in apoptotic cells. In lysates from apoptotic cells, the cleavage of U1-70 kDa is potently inhibited by Ac-DEVD.CHO but not by Ac-YVAD.CHO, strongly suggesting that caspase-3 or possibly caspase-7 is the major caspase responsible for its cleavage in apoptosis. Cleavage of U1-70 kDa separates the RNA binding domain from the distal arginine-rich region of the molecule, which may have a dominant-negative effect on splicing; such inhibition of splicing would block cellular repair pathways dependent on new mRNA synthesis [105].

Fodrin

During the execution phase of apoptosis, a number of important plasma-membrane changes occur, resulting in the recognition and subsequent phagocytosis of the apoptotic cell either by a professional phagocyte or by a neighbouring cell. Cleavage of important cytoskeletal proteins, including actin [124,125], Gas2 [126] and fodrin (non-erythroid spectrin) [127-129], during apoptosis may induce cell shrinkage and membrane blebbing, and alter cell survival signalling systems. α -Fodrin, an abundant membrane-associated cytoskeletal protein, is rapidly and specifically cleaved during CD95- and TNF-induced apoptosis, and this appears to be related to the membrane blebbing. Initially it was proposed that fodrin is cleaved by calpain I [127], but the cleavage is probably due to a caspase [128,129]. Fodrin contains several potential caspase cleavage sites, including a DETD[S] site only nine amino acids away from the proposed calpain cleavage site, which may have led to the initial confusion [128]. Treatment of cells with Ac-DEVD.CHO protected them from CD95-induced apoptosis, but did not prevent the proteolysis of fodrin [128], suggesting that fodrin proteolysis can be uncoupled from apoptosis and that it is mediated by a caspase other than caspase-3. If correct, this is an intriguing observation, as it suggests that some caspases (those responsible for the cleavage of fodrin) may be activated and yet apoptosis may still be prevented. An alternative possibility is that Ac-DEVD.CHO may have inhibited only some features of the apoptotic phenotype, including those used to assess apoptosis in the particular study [128]. The inhibitory characteristics of the caspase responsible for fodrin proteolysis, i.e. readily inhibited by Ac-YVAD chloromethyl ketone and relatively insensitive to Ac-DEVD.CHO, are reminiscent of caspase-6.

Protein kinase C δ and Rb

Protein kinase C δ is also specifically cleaved during apoptosis to a catalytically active fragment by caspase-3, but not by caspases-2, -4, -5, -6 and -7. Protein kinase C δ is one of the few examples of a substrate that is cleaved by caspase-3 but not by caspase-7. Interestingly, overexpression of this fragment, but not of full-length protein kinase C δ or a kinase-inactive fragment, is associated with chromatin condensation, nuclear fragmentation and cell death, suggesting that the proteolytic activation of protein kinase C δ may contribute to certain features of the apoptotic phenotype [130,131].

Rb is an important mediator of cell cycle progression and regulation. Phosphorylation of Rb by cyclin-dependent kinases inactivates its growth-suppressive functions and drives cells through the cell cycle into mitosis. More recently, an anti-apoptotic function of Rb has also been described [132]. Several groups have described the cleavage of Rb during apoptosis [111,133,134]. Interestingly, different-sized cleavage products of Rb were observed in these studies, suggesting the possible

involvement of different caspases. In one study, the cleavage site of Rb was identified as a DEAD[G] motif in the C-terminal region of the molecule, suggesting the likely involvement of caspase-3. The cleaved Rb failed to bind the regulatory protein Mdm2, which may lead to apoptosis due to inactivation of Rb functions [111].

INHIBITION OF CASPASES BY CrmA, Bcl-2 FAMILY MEMBERS, p35 AND PEPTIDE INHIBITORS

CrmA

When ectopically expressed, the *crmA* gene prevents apoptosis in a number of different systems [21,22,58,92,135-137] (Table 4). However, the ability of CrmA to inhibit apoptosis is clearly dependent on the stimulus used to induce it (Table 4). Thus there are CrmA-sensitive and -resistant pathways present in the same cell types. The recognition motif cleaved within CrmA is LVAD [138]. CrmA was originally described as a novel specific inhibitor of caspase-1 [45], but it is now apparent that it differentially inhibits different caspases. CrmA is a protease inhibitor that inhibits cell death, most probably by inhibiting one or more caspases. CrmA is a poor inhibitor of CED-3, caspase-2, caspase-3, caspase-7 and caspase-10, but an effective inhibitor of caspases-1, -4, -6 and -8 (Table 1) [58,65,67,83,84,135,138,139]. CrmA blocks both CD95- and TNFR-1-mediated cell death at much lower concentrations than are required for the inhibition of caspase-3 and caspase-7 [23,49,50,64,85]. Caspase-3 and caspase-7 are proteolytically activated following stimulation with CD95 or TNFR-1, but remain as zymogens in anti-CD95-treated CrmA-expressing cells, suggesting that CrmA inhibits a protease upstream of caspases-3 and -7 [85,140]. The effects on caspases-8 and -10 may be particularly important, as both of these caspases are capable of processing themselves and all other known caspases [67], and caspase-8 appears to be at the apex of the apoptotic cascade induced by CD95 or TNF [96,97]. Caspase-8 is very sensitive to inhibition by CrmA, whereas caspase-10 is only poorly inhibited, requiring a ~1000-fold greater concentration of CrmA to give the same inhibition [67]. Thus the sensitivity of CD95-induced apoptosis to inhibition by CrmA appears to be due to inhibition of caspase-8. Overexpression of caspase-8 results in apoptosis, which is inhibited by CrmA, suggesting that caspase-8 may be the physiological target of CrmA or, alternatively, that there is a downstream CrmA-

Table 4 Bcl-2 and CrmA regulate different pathways

Apoptosis induced in the same cells by different stimuli is often CrmA-sensitive or CrmA-resistant. The former pathways are frequently Bcl-2/Bcl-x_l-resistant, while the latter are Bcl-2/Bcl-x_l-sensitive. Abbreviations: NGF, nerve growth factor; ND, not determined; Ara-C, Cytarabine.

Treatment	Cells	CrmA-sensitive	Bcl-2/Bcl-x _l -sensitive	References
Mouse ICE	Rat-1	+++	+++	21
CED-3	Rat-1	+	+	21
Caspase-2	Rat-1, HeLa	+	+++	58,135
NGF withdrawal	Dorsal ganglion neurons	++	++	22
CD95	Jurkat, BJAB, MCF7	+++	-	135,140
Staurosporine	Jurkat T cells	-	+++	140
CD95	Lymphoma cells	+++	-	136
γ -Irradiation	Lymphoma cells	-	+++	136
CD95	U937	+++	ND	137
Ara-C	U937	-	+++	137
TRADD	HepG2, NIH 3T3	+++	-	92

sensitive protease. These results further support the suggestion that caspase-8 lies at the apex of the apoptotic cascade triggered by CD95 and TNF, and explain why CrmA is such a potent inhibitor of apoptosis resulting from these stimuli (Figure 4). In contrast, CrmA-insensitive pathways may involve caspase-10 or a closely related homologue as their most upstream caspase (Figure 4).

Bcl-2 family

Bcl-2 is the mammalian homologue of CED-9, which is a negative regulator of CED-3 [19]. Bcl-2 and related family members, such as Bcl-x_L, inhibit cell death induced by many stimuli [141,142]. Interestingly, many of the apoptotic pathways that are sensitive to inhibition by CrmA are relatively resistant to Bcl-2/Bcl-x_L, and vice versa (Table 4). These findings suggest the presence of a CrmA-sensitive (CD95)/Bcl-x_L-insensitive and a CrmA-insensitive/Bcl-x_L-sensitive pathway for the induction of apoptosis [137]. As discussed earlier, specific interactions of TRADD and FADD with an intracellular domain of TNFR-1 and CD95 respectively induce apoptosis, and both of these are inhibited by CrmA, whereas neither Bcl-2 nor the *E1B* gene product block TRADD-induced cell death [91,92]. The CrmA-sensitive pathways generally appear to involve plasma membrane receptor-mediated apoptosis, compatible with the inhibition of a specific caspase (such as caspase-8) being recruited to the cell membrane. Bcl-2/Bcl-x_L seems relatively ineffective at inhibiting this type of receptor-mediated apoptosis, but more efficacious than CrmA at inhibiting other forms of apoptosis.

Several recent studies suggest that Bcl-2 and Bcl-x_L exert their anti-apoptotic action at or before the processing of certain caspases to their catalytically active forms [140,143–145]. For example, overexpression of Bcl-2 or Bcl-x_L prevents staurosporine-induced cell death of Jurkat T cells and the processing of both caspase-3 and caspase-7, placing these negative regulators of apoptosis at or upstream of the processing of caspases-3 and -7 [140].

p35

The baculovirus *Autographa californica* p35 gene product inhibits apoptosis in insects, mammals and nematodes, suggesting that it acts at a central and evolutionarily conserved part of the apoptotic pathway [24,138,146,147]. Purified recombinant p35 inhibits the activity of purified recombinant caspases, including caspases-1, -2, -3 and -4, and maximum inhibition is achieved at equimolar concentrations of p35 and the caspase. Cleavage of p35 by a caspase results in the formation of a caspase-p35 complex. The presence of such complexes *in vivo* would prevent the caspases from initiating the apoptotic cascade. The CED-3 cleavage site in p35 is DQMD[G, which is required for protection against programmed cell death in the nematode. Interestingly, CrmA does not protect against cell death in the nematode unless its caspase-1 cleavage site (LVADC) is replaced by the CED-3 cleavage site in p35 [138].

Peptide inhibitors

In addition to the reversible inhibitors Ac-DEVD.CHO and Ac-YVAD.CHO, use of the irreversible tripeptide caspase inhibitor Z-VAD.FMK [benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone] has helped to elucidate the role of caspases in apoptosis, in particular in intact cellular systems. Z-VAD.FMK is a cell-permeable irreversible inhibitor of caspases whose permeability is facilitated by the presence of the benzyloxycarbonyl and OMe groups. Z-VAD.FMK is a potent inhibitor

of apoptosis induced by a wide range of stimuli in a number of different systems, including thymocytes, hepatocytes, human monocytic THP.1 cells, Jurkat T cells, neuronal cells and *Drosophila melanogaster* [72,144,148–153]. Z-VAD.FMK also suppresses programmed cell death in the interdigital webs of developing mouse paws, suggesting that it may be of value in studying developmental cell death [72,154]. Z-VAD.FMK inhibits apoptosis at an early stage, as judged by its inhibition of all the ultrastructural features of apoptosis, i.e. PARP cleavage, processing of caspase-3, formation of large fragments and internucleosomal cleavage of DNA [71,72,149,150]. More recently we have shown that it inhibits the processing of caspases-2, -3, -6 and -7, suggesting that it inhibits a caspase at or near the apex of the apoptotic cascade [62]. In this regard, it is of interest that Z-VAD.FMK blocks apoptosis induced by caspase-8 [97]. Many, but not all, apoptotic cell deaths are inhibited by Z-VAD.FMK [155,156]. Whereas it completely inhibited apoptotic death induced by different stimuli in thymocytes [150,155], it had a variable effect on cell death in peripheral T cell blasts while complete sensitivity to another caspase inhibitor was maintained, suggesting the activation of different caspases at different stages of T-cell maturation [155]. Z-VAD.FMK did not inhibit Bax (a Bcl2 family member)-induced cell death, although it did inhibit certain features of the apoptotic phenotype, including cleavage of nuclear and cytosolic substrates and DNA fragmentation, suggesting that Bax-induced cell death may not require caspases [156]. *In vivo*, Z-VAD.FMK prevents the normally fatal liver damage induced by anti-CD95 injection [157]. Activation of both caspase-1-like and caspase-3-like protease activities is detected in liver lysates, as is the cleavage of caspase-3 to its p17 fragment. Repeated injections of Z-VAD.FMK results in the complete survival of all animals, with no histopathological signs of liver damage. The treatment with Z-VAD.FMK does not prevent the initial early small rise in ICE-like activity, as assessed by Z-YVAD-T-amino-4-trifluoromethylcoumarin cleavage; however, it does prevent processing of caspase-3 and the increase in caspase-3-like protease activity, as assessed by Z-DEVD-T-amino-4-trifluoromethylcoumarin cleavage. This and other observations suggest that non-toxic caspase inhibitors may have potential clinical applications in fulminant hepatitis arising from viral hepatitis [157] and various neurodegenerative disorders [5,154].

A HIERARCHY OF CASPASES

All caspases are cleaved at specific Asp residues, raising the possibility that some caspases sequentially activate others, so establishing a hierarchy of caspases. Such a model has been proposed in which caspase-8 has been termed an 'initiator' protease, which activates an 'amplifier' protease such as caspase-1, which in turn activates a 'machinery' protease such as caspase-3 or caspase-7 [158]. Much of the evidence for this, and the concept of a hierarchy of caspases, is based on *in vitro* data with recombinant enzymes; the limitations of such approaches have already been discussed. It is important, where possible, to determine if such a cascade of caspases occurs in cells undergoing apoptosis. PARP cleavage generally precedes lamin cleavage in cells undergoing apoptosis [68,87,115,116]. As caspase-3 and caspase-7 cleave PARP but not lamins, and caspase-6 cleaves lamins, this suggests that, in cells undergoing apoptosis, activation of caspase-3 and caspase-7 precedes activation of caspase-6 (Figure 4). In human monocytic tumour cells undergoing apoptosis, processing of caspases-2, -3, -6 and -7 was observed, although the precise sequence of activation of these caspases was not determined [62].

Different structural features, such as specific cleavage-site motifs, in the different caspases may give clues to their activities and to which caspase lies upstream of others (Table 1; Figures 2 and 3). The presence of a DXXD motif in caspases-2, -6 and -9 (Table 1) suggested that these three caspases may be activated by caspase-3, and this has been substantiated by *in vitro* experiments. A WXXD motif occurs in all three members of the ICE subfamily (Table 1), suggesting that they may be activated by the same caspase. Similarly, IXXD and LXXD motifs recur in different caspases (Table 1), supporting the notion that there may be a small number of key caspases that activate other family members. Good candidates for such critical caspases are caspase-8 and caspase-10, because they activate all other caspases and also contain FADD-like prodomains, which permit extensive protein-protein interactions [66,67,96,97]. Stimulation with either CD95 or TNF results in the recruitment and activation of caspase-8, supporting the hypothesis that it lies at the apex of the apoptotic cascade following specific receptor-mediated activation (Figure 4). Other types of receptor-mediated activation may involve other caspases, such as caspase-10. It has been proposed that caspase-10 may be involved in many CrmA-insensitive non-receptor-mediated cell deaths, as it is poorly inhibited by CrmA [67]. Once activated, some family members may then further activate the caspase that initially caused their activation, so setting up a caspase amplification cycle (Figure 4) [82]. The presence of CrmA-sensitive and -insensitive pathways also suggests that not all cell death pathways utilize the same caspases.

While many intracellular targets of the caspases have been recognized (Table 3), the critical cellular substrates leading to cell death have not yet been identified. The mechanisms by which caspases are regulated in cells in order to prevent their unwanted demise, and the possible role of apoptosis-inhibitory proteins, will be the subject of intense investigation. The long prodomains of some caspases, such as caspase-2, may enable them to be recruited by adapter molecules such as RAIDD/CRADD, so facilitating the execution of the cell death programme [101,102]. It remains to be determined whether there is a family of RAIDD/CRADD-like molecules that can recruit other caspases with prodomains.

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